THE KINETICS OF METABOLISM IN THE PERFUSED HEART

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ABSTRACT OF THESIS

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The kinetics of the permeation of cardiac muscle cells by glucose and the effect of insulin on this process were examined in the isolated perfused rat heart. Previous attempts to determine the parameters of permeation by glucose are considered to be imprecise because endogenous insulin influenced experimental results, intracellular glucose was inaccurately measured and estimates of glucose utilisation were not associated with defined concentrations of extracellular or intracellular glucose.

In principle the parameters of permeation can be determined from a steady state relationship between glucose uptake and the intracellular and extracellular concentration of glucose. Alternatively they can be determined from the relationship between the uptake and the extracellular concentration of glucose. if the kinetics of glucose metabolism are taken into account.

A novel apparatus for cardiac perfusion was therefore developed in which hearts were brought to a steady state of glucose permeation and utilisation. In the apparatus hearts were perfused with a small volume of perfusate under well controlled conditions. Glucose concentration in perfusates was estimated by rapid and accurate automated methods which met the requirements of the procedure. The utilisation of glucose was determined not only in the steady state but also throughout the approach to that state.

Use other side if necessary.

The first method for the determination of the parameters of permeation was impracticable because accurate estimates of intracellular glucose could not be made with methods currently available. Consequently the kinetics of glucose utilisation were compared with the predictions of a mathematical model in which permeation was assumed to be a simple carrier mechanism and the phosphorylation of glucose to be an irreversible enzyme-catalysed reaction. Agreement was found. Estimates made of the parameters of permeation in the presence and absence of insulin were in qualitative accord with previous work. The hormone increased the half saturation constant at least 3-fold and the maximum rate of permeation 5-fold.

The estimates of the parameters of permeation in the presence of insulin may be inaccurate because in their determination the possibility of a concentration gradient in the extracellular glucose was ignored. However, the estimates provide a reasonable explanation of a phenomenon which was observed when the time-course of glucose utilisation was studied in the absence of exogenous insulin. A stimulation of utilisation, presumably by endogenous insulin, occurred consistently only at concentrations of glucose greater than 1mM.

It was concluded that the estimates are an improvement on previous values and that the apparatus is a useful addition to the methods available for the study of the metabolism of isolated hearts.

DECLARATION OF ORIGINALITY

I declare that this thesis was composed

by myself and that the research which is described was mine.

J.A. O'Brien

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GENERAL INTRODUCTION

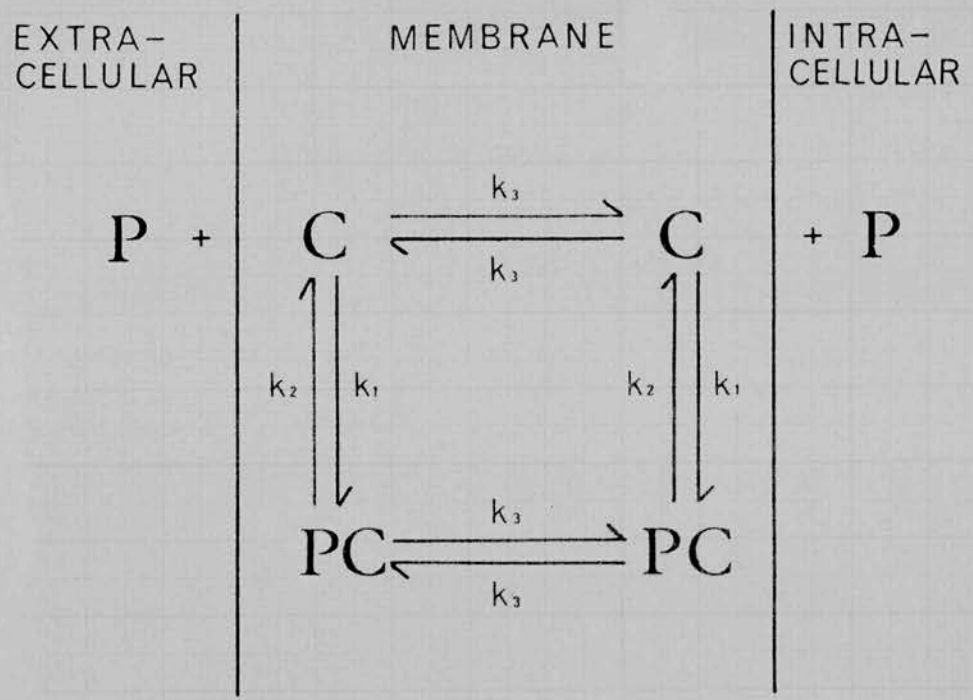
The object of the work presented in this thesis is the investigation, under steady-state conditions, of the kinetics of the permeation of glucose into cardiac muscle cells of rats and the examination of the effect of the hormone insulin on this process. Where relevant, experimental data have been tested against the predictions of the hypothesis that the transfer of glucose across the membranes of muscle cells is mediated by combination with some component of the membrane, commonly referred to as a carrier.

The Carrier Hypothesis

The concept of the "facilitation" by carriers of the transfer of some electrolytes and non-electrolytes across cell membranes has largely developed from studies of the permeability of the erythrocyte. Theories of permeation based upon relationship between the penetration rate and the lipid solubility (Overton, 1895; Hedin, 1897) and molecular size of the permeant (Höber and Ørskov, 1933) proved inadequate for the interpretation of the properties of the penetration of cells by monosaccharides. The kinetics of simple diffusion are not obeyed, because the value of the apparent diffusion coefficient decreases as the extracellular concentration of a sugar is raised (Wilbrandt, Guensberg, and Lauener, 1947). As this observation suggests, the process becomes saturated at high concentrations of the permeant and its kinetic properties can be formulated in the manner of the theory applied by

Figure 1.

A MODEL OF CARRIER-MEDIATED PERMEATION



P = permeant

C = carrier

PC = permeant-carrier complex

k = rate constant

Michaelis and Menten (1913) to the catalysis of the hydrolysis of sucrose by yeast invertase. Another characteristic of the process is its specificity towards the permeant. This feature of permeation was early indicated by the observation of Hedin (1897) that inositol, a polyhydric alcohol of the same molecular weight as the hexoses, does not enter red cells. In contrast, monosaccharides penetrate red cells but do so at different rates (Kozawa, 1914). The specificity of the process has since been more clearly defined. Not only do the affinities of monosaccharides for the system vary in their order of magnitude but also the D-isomers of hexoses, for example, permeate red cells while the L-isomers are virtually excluded (LeFevre and Marshall, 1958). Furthermore, the components of mixtures of monosaccharides do not behave independently. Each inhibits the permeation into cells of the others (LeFevre and Davies, 1951). From these facts, it would seem that many monosaccharides permeate into red cells by a common, saturable process.

It was this and other related evidence which led to the development of the concept of the permeation of cell membranes by sugars by a three step process. The permeant combines with a carrier, the complex formed diffuses across the membrane, the complex dissociates at the other face of the membrane. Fig. 1 illustrates the most simple scheme for such a process, and indicates the assumptions made in deriving the kinetics appropriate to it. The interaction between sugar and carrier is presumed to be first order, with respect to the sugar, and to be freely reversible such that the association and dissociation constants, k_1 and k_2, have the same value at both faces of the membrane. It is also supposed that the diffusion coefficient, k_3, of the sugar-carrier complex in the membrane is equal to that of the free carrier.

With the further assumption that the diffusion coefficient is smaller than the association and dissociation constants and that therefore the permeant may be assumed to be in equilibrium with the carrier, the equations defining the kinetic properties of this model have been derived by Widdas (1952) and Rosenberg and Wilbrandt (1955).

In this work, the basic equation, which defines the net rate of permeation, v, has been taken to be:-

$$v = \frac{VK(x - y)}{(K + x)(K + y)}$$

This equation is derived in Appendix 1. p186. x and y represent the con-concentrations of the extracellular and intracellular permeant respectively. V, although not identical with "Vmax" of enzyme kinetics, has a similar significance in that it defines the maximum rate of permeation. K, which will be referred to as the half-saturation constant, is analogous to Km. Despite the similarities which exist between the theories of carrier-mediated permeation and enzyme-catalysed reactions, in order to stress the distinction of the two, the terminology and conventions of the latter have not been wholly adopted.

As indicated in Appendix 1, the equation is an approximation which is fully valid only if the rate-limiting step in permeation is the transfer across the membrane rather than the dissociation of the complex. The kinetics of the permeation of glucose in red cells have been interpreted as justifying the assumption (Widdas, 1953, Britton, 1964).

A prediction of the carrier hypothesis is that the transfer of a sugar

against a concentration gradient may be induced, independently of metabolic energy, by the transfer of a competing sugar in the opposite direction. This phenomenon of counter-transport was observed in rabbit erythrocytes where the influx of glucose induced the efflux of xylose against a concentration gradient (Park, Post, Kalman, Wright, Johnson and Morgan, 1956).

The characteristics of the transfer of sugars across the membrane of erythrocytes are also to be observed in muscle tissues. Fisher and Lindsay (1956) and Bronk and Fisher (1957) showed that the penetration of D-galactose into the perfused rat heart does not conform to the kinetics of simple diffusion and that the penetration is inhibited by the presence of D-glucose. These observations on cardiac muscle were extended (Fisher and Zachariah, 1961) to demonstrate the reduction in the rate of penetration of the pentoses, D-xylose and L-arabinose, by D-glucose or D-galactose. Morgan, Cadenas and Park (1960) also showed competition between D-glucose, D-3-O-methylglucose, D-xylose and L-arabinose in permeation of cardiac muscle and in addition the counter-transport of D-3-O-methylglucose induced by a glucose gradient. Similarly, competition among monosaccharides occurs in their permeation of diaphragm muscle (Battaglia and Randle 1959, 1960). Consequently, it is the general practice to interpret the kinetics of sugar transfer across the membranes of muscle cells in terms of the carrier hypothesis.

The validity of the assumptions made in the simple form of the carrier hypothesis has been questioned. Mawe and Hempling (1965) and Levine, Oxender and Stein (1965) observed that the efflux of isotopically labelled glucose from erythrocytes into saline media is faster when the medium contains unlabelled glucose, supposedly at the intracellular concentration, than when

no extracellular glucose is present. These authors concluded that the rate constant for the transfer of the glucose-carrier complex between the surfaces of the membrane is greater than that for the free carrier. However, Miller (1968) argued that the assumption of a high rate of transfer for the complex does not provide an adequate quantitative description of the phenomenon of counter-transport. Miller also considered the implication of a rate of diffusion of the permeant from the bulk of the extracellular and intracellular water to the surface of the membrane which is of the same order as the rate of transfer of carrier or complex across the membrane. He found that this theory has qualitative attractions but is still quantitatively inadequate. The problem remains essentially the difficulty of explaining the observation that the rate of transfer of a sugar across a membrane is a function of the concentration of the same or another sugar on the opposite side of the membrane. It may be significant that net transfer of sugar must be accompanied by bulk flow of water which will presumably affect the integrity of an unstirred layer of water supposed to exist about an erythrocyte (Sha'afi, Rich, Sidel, Bossert, & Solomon, 1967) when there is no net transfer of sugar and of water.

In the case of muscle cells, there appears at present, to be no intimation of added complexity, although this may well be masked by the comparative crudity of data obtained from such sources, whether owing to technical difficulties or to the greater impact of biological variations. On the other hand, approximations which are valid in their application to permeation of erythrocytes may not apply to permeation in muscle tissue. However, no such distinctions have been reported.

It should be stressed that not all authors accept that the properties of

permeation are determined by the nature of cell membranes and they therefore reject the carrier hypothesis. The hypothesis they offer is one in which the properties of permeation are those of a process of diffusion determined by the bulk of the cytoplasm (Ling, 1956; Fenichel and Horowitz, 1963): Fenichel and Horowitz (1963) have analysed the kinetics of the efflux from frog sartorious muscle of amides, alcohols, and thioureas in terms of those of bulk diffusion and found them to agree. However, there seems to be a weakness in their experimental approach. They used only one concentration of each permeant in their studies whereas a true test of the correspondence of their data to the kinetics of bulk diffusion would be to show that the apparent value of the diffusion coefficient is independent of the concentration of the permeant. That this is not so for the penetration of human erythrocytes by monosaccharides has been demonstrated by Fisher and Nimmo (personal communication). Until the proponents of bulk diffusion can produce evidence of a correspondence of their data to their theory over a range of concentrations of permeant, and a convincing explanation of the competitive nature of permeation, the carrier hypothesis remains that best suited to the evidence.

The Effect of Insulin on the Permeation of Monosaccharides

The hormone insulin has a striking effect upon the uptake of glucose by muscle and adipose tissue. Its action complicates the problem of the permeation of monosaccharides into muscle. Whatever hypothesis is proposed to explain the features of glucose transfer into muscle, it must necessarily cover the influence of insulin on this tissue. A brief review follows of the evidence which associates insulin with the permeation of sugars into

A pancreatic factor involved in the regulation of the concentration of glucose in the blood was indicated by the diabetic condition induced by pancreatectomy (von Mering and Minkowski, 1889). The elucidation of the properties of this factor, named "insuline" by de Meyer in 1909, was largely denied until its isolation by Banting and Best (1922). Within a few years of the isolation, an effect of insulin on the carbohydrate metabolism of muscle was proved; indeed this can be said to be one of its earliest recognized actions. The hormone was found to increase the rate of utilisation of glucose by the isolated perfused rabbit heart (Hepburn and Latchford, 1925) and the rate of glucose oxidation and glycogen formation in the muscles of the decapitated, eviscerated cat (Best, Dale, Hoet and Marks, 1926).

The subsequent four decades of research on the influence of insulin on carbohydrate metabolism has revealed a diversity of effects without achieving a complete clarification of their interrelation. Leaving aside the whole question of the action of insulin on carbohydrate metabolism in liver where the permeation of glucose is so rapid that any physiological significance of an effect of insulin on this process can be discounted, the great majority of work has been with three preparations from the peripheral tissues: the isolated rat diaphragm, the perfused heart and the epididymal fat pad. The introduction by Gemmill of the use of the isolated diaphragm with its evident advantages over a tissue slice or homogenate has been a major influence in this field. A stimulation by insulin of glycogen deposition described by Gemmill (1940) has been exploited for the assay of insulin. However, a promotion of glucose oxidation in the diaphragm by insulin remains the subject of controversy.

Chain and his colleagues (in work summarised by Chain, Beloff-Chain and Pacchiari, 1956) in a detailed and elegant radiochromatographic analysis of the fate of labelled glucose in the isolated diaphragm, distinguished between the pattern of the metabolism of glucose when promoted by the action of insulin or by increases in the concentration of glucose in the medium. Insulin caused no increase in the rate of carbon dioxide or lactic acid formation: but such increases were effected by glucose in high concentrations. In both cases, glycogenesis was stimulated. These observations were taken to indicate an intracellular action of insulin on the pattern of carbohydrate metabolism in the diaphragm. On the other hand, Villee, Deane and Hastings (1949) claimed that insulin stimulates the formation of carbon dioxide from labelled glucose in the isolated rat diaphragm.

These differences in conclusions among workers may be attributable to deficiencies in the preparation which they studied. Since it appears that each muscle cell in the rat diaphragm has one attachment to the rib cage and the other to the central tendon, the cut diaphragm consists entirely of damaged cells which may lose, to the extracellular water and medium, enzymes in amounts sufficient to effect the metabolism of glucose outside the cells (Candela, Sols, Alvarado, Santiago, Villar-Palasi and Candela, 1957; Zierler, 1956). Disturbances in the proportions of intracellular and extracellular transformation of glucose might explain the observations of Chain et al. (1956).

The conflict of opinion on the influence of insulin on diaphragm muscle has not extended to the action of the hormone on glucose metabolism in the epididymal fat pad. In this preparation, as in muscle tissues, insulin stimulates glycogenesis (Renold, Marble and Fawcett, 1950). Of greater sig-

nificance is the conclusion of Winegrad and Renold (1958 a,b) that the action of insulin on glucose U-^{14}C, glucose 1-^{14}C and glucose 6-^{14}C was to stimulate equally the phosphogluconate and Embden-Meyerhof pathways.

The evident differences in the physiological roles of muscle and adipose tissues and in the interaction of fat and carbohydrate metabolism in them preclude the assumption that the action of insulin on one should serve as a model for its action on the other. Although, in one tissue or another, stimulation by insulin of all the possible transformations of glucose has been claimed, doubt remains whether all the actions of insulin on carbohydrate metabolism are due to its effect on a single process early in the transformation of glucose. To be common to all the pathways affected by insulin, such a process could only be the entry of glucose into the cell or its phosphorylation to glucose-6-phosphate.

Evidence that insulin could remove an inhibition of hexokinase brought about by an anterior pituitary factor was presented by C.F. Cori, G.T. Cori, and their collaborators (Price, Cori and Colowick, 1945; Price, Slein, Colowick and Cori, 1946; Colowick, Cori and Slein, 1947). The inability of other workers to confirm this effect (Broh-Kahn & Mirsky, 1947; Stadie & Haugaard, 1949) and the observation that insulin has as large an effect *in vitro* on the diaphragm of hypophysectomised rats as on that of normal rats (Perlmutter and Greep, 1948) have weakened the status of the hexokinase hypothesis. These contradictory results might reflect the operation of unknown factors. There is, for instance, a body of evidence which points to the presence in the serum of human diabetics (Weil-Malherbe and Bone, 1951) and of diabetic-hypophysectomised rats, injected simultaneously with pituitary

growth hormone and cortisone, of a lipoprotein inhibitory towards hexokinase (Bornstein and Park, 1953) even under in vitro conditions (Krahl and Bornstein, 1954). Furthermore, this lipoprotein depresses the uptake of glucose by diaphragm muscle and diminishes the stimulating action of insulin upon the preparation (Bornstein, 1953). There is therefore a conflict of evidence, which may reflect the complexity of the diabetic state. Many of the effects of diabetes are only slowly reversed by insulin in contrast to the speed of the hypoglycaemic action of the hormone. It would seem that the hexokinase hypothesis cannot be valid as originally stated and may need considerable adjustment to embrace these new facts.

The alternative hypothesis that insulin might increase the rate of entry of glucose into cells was suggested by Höber (1914). Since differences among species in the permeability of erythrocytes could reflect differences in the constitution of their plasma membranes, he proposed that changes in the normal constitution of membranes might explain pathological states, particularly diabetes. Evidence in support of such a theory was not forthcoming until Pollak and Fehér (1936) showed that injection of insulin into the rat resulted in an increase in the intracellular concentration of galactose in the heart and diaphragm. Later, Lundsgaard (1939) concluded that the transfer of glucose across the membranes of skeletal muscle cells was the limiting process in the metabolism of the sugar. He argued that an increase in glucose utilisation in the presence of insulin must reflect an increased availability of glucose for metabolism.

These early observations provided a basis for the hypothesis of a stimulation by insulin of the permeation of sugars into muscle cells. This

basis was consolidated by Levine who studied the volume of distribution of non-metabolised or poorly metabolised monosaccharides in eviscerated, nephrectomised animals (Levine, Goldstein, Klein and Huddlestun, 1949; Levine, Goldstein, Huddlestun and Klein, 1950). For instance, the distribution of galactose is normally throughout 45% of the fluid volume of this preparation. In the presence of insulin, it rose to 75%. This observation was confirmed in the eviscerated rabbit by Wick and Drury (1953).

Park and Johnson (1955) determined the intracellular content of glucose and galactose in the diaphragm and heart of normal, eviscerated and alloxan diabetic rats given continuously an infusion of the sugar, with and without insulin. These experiments showed that insulin causes an increase in the intracellular concentration of the free sugars. Similar results were obtained for glucose (Park, Bornstein, and Post, 1955) and for pentoses (Kipnis and Cori, 1957) with the isolated rat diaphragm. In the isolated perfused rat heart preparation, developed by Bleehen and Fisher (1954), insulin increases the intracellular concentration of glucose and galactose (Fisher and Lindsay, 1956).

Had a stimulation of hexokinase by insulin occurred in the experiments described above, a diminution of the concentration of intracellular glucose might have been expected, and certainly not an increase; no effect on non-metabolised sugars would be predicted. The evidence is clearly consistent with the view that insulin promotes the entry of some monosaccharides into the muscle cell. If the carrier hypothesis for the permeation of sugars into muscle cells is valid, it must account for an effect of insulin on this process.

The Action of Insulin on the Kinetics of Sugar Permeation

For much of the work on the kinetic aspects of the effect of insulin on permeation, the isolated perfused rat heart has been the preferred preparation. There are several reasons for this choice. The viability of a perfused heart can, to some extent, be assessed by observation of such factors as the rate and force of contraction. A permeant and a hormone can be presented to the cells by the physiological route of the coronary arteries and smaller vessels. In its preparation, the heart suffers little damage which might lead to subsequent loss of enzymes.

Reference has already been made to the loss of enzymes from the isolated diaphragm. In this respect, the intact diaphragm preparation, in which the origins of the muscle fibres on the ribs of the animal remain whole, would be expected to be superior to the cut diaphragm. Nevertheless, Zierler (1956) believes that aldolase can escape across the boundaries of intact cells in the diaphragm. In the perfusate of the isolated rat heart, Fisher and Shipp (personal communication) were unable to detect more than a trace of aldolase. There is no evidence that such loss of aldolase and other enzymes results in a significant rate of extracellular metabolism of glucose.

The inability to perfuse the vascular system of the diaphragm raises the possibility that diffusion from the medium to the muscle cells might constitute an unphysiological rate-limiting step when the permeation of a sugar into the cells is a rapid process. Sucrose requires 15 minutes to become virtually equilibrated with the extracellular fluid of the intact diaphragm from a rat (Norman, Menozzi, Reid, Lester and Hechter, 1959) and, with a half-time of about 6 seconds, requires only 45 seconds in the isolated rabbit heart

TABLE 1

EFFECT OF INSULIN ON THE PARAMETERS OF THE PERMEATION OF D-XYLOSE AND L-ARABINOSE IN RAT CARDIAC MUSCLE

(From Gilbert, 1963)

	D-Xylose		L-Arabinose	
Insulin mU./ml.	K mM.	V mMoles/l ICW/min.*	K mM.	V mMoles/l ICW/min.*
0	0.17	1.47	0.05	2.16
0.2	5.49	1.03	1.41	1.11
1.0	6.69	4.75	1.23	4.12
4.0	7.08	30.06	2.23	30.87
20.0	6.42	217.70	1.62	165.60

*Units for rate of permeation: mMoles per litre of intracellular water per minute.

perfused through the coronary circulation (Schafer and Johnson, 1964). If the rate of passage through the extracellular water of a perfused heart can limit the uptake of a permeant by the muscle cells, this restriction has the merit of being relevant to the in vivo state.

Fisher and Zachariah (1961) and Fisher and Gilbert (Gilbert, 1963) examined the effect of insulin on the parameters of the permeation of non-metabolised sugars in the isolated perfused rat heart. They followed the approach to equilibrium between the influx and efflux of D-xylose and L-arabinose. The carrier hypothesis predicts a relationship between time, t, and the ratio, f, of the intracellular concentration, y, to the extracellular concentration, x, of a sugar such that:

$$f/t = -\frac{K + x}{x} \cdot \frac{\ln(1 - f)}{t} - \frac{KV}{x(K + x)}$$

In practice, the equation is modified to take into account changes in the intracellular volume due to osmotic effects during the approach to equilibrium. Confirming and extending the results of Fisher and Zachariah (1961), Gilbert (1963) obtained the values for the parameters of the permeation of D-xylose and L-arabinose shown in Table 1. Insulin increases the K for the permeation of both sugars. The K is apparently independent of the concentration of insulin used. In contrast, the V is sensitive to the concentration of insulin and is depressed at a low concentration of the hormone but raised progressively at higher concentrations. Although the estimates have a large error, which will be discussed in Section IV, it seems probable that the trends which they suggest are real.

An increased K implies that a higher concentration of sugar is necessary to saturate the postulated carrier. Consequently, in the presence of insulin a higher intracellular concentration of a sugar is possible before efflux becomes maximal. For glucose, a similar effect of insulin in raising the intracellular concentration of this metabolised sugar would also result in a higher rate of phosphorylation, provided that the hexokinase remains unsaturated.

The variations in V with increasing levels of insulin cannot be explained by invoking an effect of insulin on the diffusion coefficient (k_3) alone. Gilbert (1963) postulated that these changes in V reflect the progressive modification of the total carrier into an insulin-carrier complex. Under the conditions of his experiments, the unmodified carrier with the lower K would rapidly become saturated by the permeant at both faces of the cell membrane and would not contribute to the net transfer of the permeant. The observed rate of permeation would then be determined solely by the properties of that fraction of the total carrier which is complexed with insulin and has the higher K. A decrease in V at a low concentration of insulin is interpreted as reflecting the small fraction of the total carrier which is effective in transferring the permeant, despite an increase in the diffusion coefficient of this fraction. When a greater fraction of the carrier is modified, at a higher concentration of the hormone, the V of the system will increase.

In terms of the carrier hypothesis, insulin might affect either k_3, the rate constant for the transfer of the carrier across the membrane, or k_2, the rate constant for the dissociation of the complex formed between the sugar and the modified carrier. Although Gilbert postulates the formation of a complex

between carrier and insulin, there is no conclusive evidence that such an interaction occurs. Since there is no certainty about the physical and chemical properties of a component of the cell membrane which could be identified with the hypothetical carrier, the nature of the interaction between insulin and carrier necessarily remains in doubt. Formation of an insulin-carrier complex is the most simple assumption consistent with the facts, but an indirect modification of the carrier mediated by insulin cannot be discounted. Provided that the amount of the carrier which is affected is assumed to be a function of the concentration of the hormone, the carrier hypothesis provides a reasonable explanation of the influence of insulin on the permeation into cardiac muscle of the non-metabolised sugars D-xylose and L-arabinose.

The Kinetics of Permeation of a Metabolised Sugar

When a permeant is metabolised, its intracellular transformation denies the possibility of following an approach to the equilibration of the influx and efflux of the permeant. The permeation of glucose into cardiac muscle cells cannot therefore be studied by the method adopted by Fisher and Zachariah (1961) and Gilbert (1963) for the investigation of the kinetics of the permeation of non-metabolised sugars. The relationship between the net rate of influx of glucose and the intracellular and extracellular concentrations of the sugar can be explored in the steady state when the net rate of influx is equal to the rate of removal of glucose by metabolism and the intracellular concentration of glucose satisfies simultaneously the kinetics of both permeation and phosphorylation. Measurement of glucose utilisation and of the intracellular and extracellular glucose concentrations in such a state

permits, through the equation

$$v = \frac{VK(x - y)}{(K + x)(K + y)}$$

derived in Appendix 1, the determination of the parameters of permeation without presuming the kinetics of phosphorylation or the effect of insulin thereon. It is clearly essential that the variables in this equation should be measured when they are functions of the same steady state and therefore interdependent. In this respect, a previous attempt by Morgan, Henderson, Regen and Park (1961) to investigate the kinetics of glucose transfer across the membranes of cardiac muscle cells was unsatisfactory.

Morgan, Henderson, et al. (1961) studied glucose permeation in the isolated rat heart perfused with medium which was recirculated in a closed system. Although the concentration of glucose in the medium must fall during such a perfusion, it can be assumed that the extracellular and intracellular concentrations of glucose in the heart pass through a continuous range of steady-state relationships. However, this experimental approach does not permit the estimation of the utilisation of glucose in the steady state which exists when the heart is taken for analysis. The uptake of glucose must be determined from the rate of decrease of the glucose concentration in a known volume of perfusate and cannot then be correlated with the perfusate glucose concentration obtaining at the cessation of perfusion.

In the experiments of Morgan, Henderson et al. (1961) the concentration of perfusate glucose decreased by at least 20% and as much as 50% during the estimation of the utilisation of glucose. The evidence of their own data

suggests that a decrease in the perfusate glucose concentration of these magnitudes must be accompanied by an appreciable diminution in the rate of glucose uptake when the concentration of perfusate glucose is insufficient to saturate the process of utilisation throughout the period of measurement. In such circumstances, the net rate of permeation of glucose in the ultimate steady state will be over-estimated. Clearly, the perfusion of a heart in a closed system has inherent disadvantages for the investigation of the kinetics of glucose permeation.

The work of Morgan and his associates can be criticised for several deficiencies other than that of their method for cardiac perfusion. Their conclusions are based on experiments in which hearts were perfused for 30 minutes without pre-perfusion beyond the elution of blood from the vessels of the freshly excised heart. Although they observed no difference in the rate of glucose utilisation measured, in the absence of insulin, over two successive periods of 15 minutes, it is probable that the permeability of the preparations to glucose varied during the perfusions. For non-metabolised sugars, the permeability of a heart, perfused in the absence of insulin, falls over the first 30 minutes of perfusion directly after the excision of the heart from the animal (Zachariah, 1961, Gilbert, 1963). The decrease in permeability can be attributed to the loss of endogenous insulin from the freshly excised heart (Zachariah, 1961). It will be shown in Section II that, without added insulin, the utilisation of glucose by a heart falls during the first 30 minutes of perfusion independently of any influence of a decreasing concentration of glucose in the perfusate.

Also of dubious validity in the work of Morgan, Henderson *et al.* are their estimations of the amount of glucose present in the heart after perfusion

and an assumption made in the calculation of the intracellular concentration of glucose. In the majority of their experiments, insufficient glucose could be detected in the heart for it to be distributed at the concentration of the perfusate glucose through a volume as large as that occupied by an extracellular marker, sorbitol. This failing was attributed in part to an inability to stop the utilisation of glucose at the end of a perfusion and in part to the presence of concentration gradients in the extracellular water. When intracellular glucose was detected, the effect of these factors in lowering the apparent concentration was ignored. On the other hand, it was assumed that only 75% of the cell water was accessible to glucose. As the cell water appears to be completely penetrated by non-metabolised sugars, (Gilbert, 1963), this assumption would give erroneously high estimates of the intracellular concentration of glucose.

The data obtained by Morgan, Henderson, et al. (1961) on the distribution and utilisation of glucose in the presence and absence of added insulin in hearts from normal animals and data obtained by Morgan, Cadenas, Regen and Park (1961) when they studied, with identical methodology, the uptake of glucose in the presence of insulin by hearts from alloxan diabetic rats were combined by Post, Morgan and Park (1961) for the estimation of the parameters of glucose permeation. The combination of data was justified by the conclusion of Morgan, Cadenas, Regen and Park (1961) that the properties of the permeation of L-arabinose in hearts from normal and diabetic animals were indistinguishable when studied in the presence of insulin, but differed when the hormone was not added to the perfusate. The latter observation can be attributed, at least in part, to a difference in the endogenous insulin of

the two preparations. Post, Morgan and Park (1961) lay claim to having made no more than a semi-quantitative estimate of the parameters of the permeation of glucose into cardiac muscle. Nevertheless, insulin at 100 mU/ml. of perfusate was reported to raise the K and V of glucose permeation in general accord with effect of the hormone on the parameters of permeation of non-metabolised sugars. An attempt to improve upon the results of these authors is justifiable not merely for the sake of precision in itself, but rather for the light which a more quantitative understanding of the permeation of glucose into cardiac muscle might cast on the regulation of carbohydrate metabolism.

The fundamental requirement for this investigation has been a technique for cardiac perfusion which will establish a stable, rather than transient, steady state of glucose permeation and will permit the determination of the utilisation of glucose in that state. In the first section of this thesis, the construction and properties of an apparatus, which has been developed to satisfy this requirement, are described. The following section is concerned with the methods for the precise automated estimation of glucose, whose use has been essential for the accurate measurement of glucose utilisation, and with the evidence for the existence of a steady state in the perfusion system. A great advantage of the technique for perfusion which has been used is that the properties of the approach of the system to a steady state enable the glucose utilisation to be studied throughout the perfusion. The time-course of glucose utilisation in the absence of added insulin reveals the metabolic instability of the preparation during at least the first 30 minutes of perfusion. The evidence presented in the second section suggests that an isolated perfused heart can attain both metabolic stability and a steady

state within an acceptable period of perfusion.

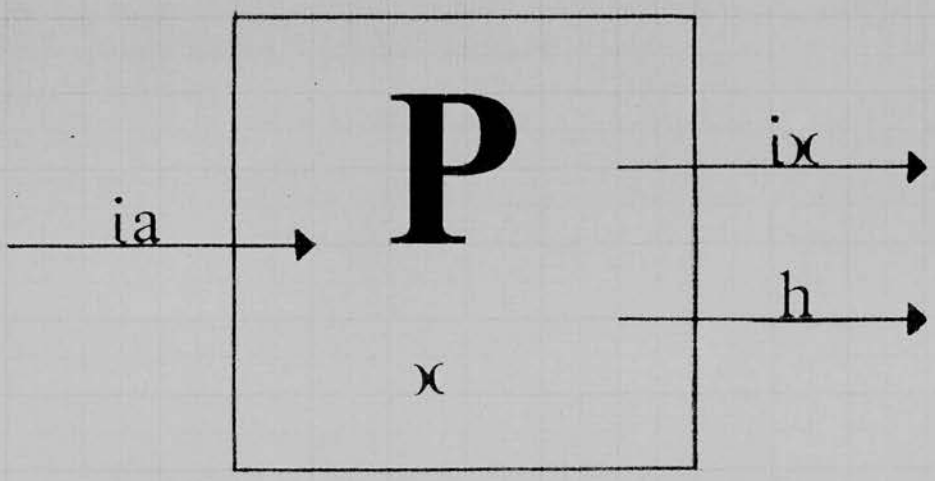
In the third section, attempts to achieve the unexceptionable determination of the intracellular concentration of glucose in a heart are discussed. The conclusion is reached that, at present, insufficient accuracy in the estimation of intracellular glucose can be attained for the estimates to be useful in the determination of the parameters of glucose permeation.

The measurement of the intracellular and extracellular concentrations of glucose in a heart when the net influx of glucose can be equated with the rate of utilisation of glucose is attractive because, in principle, the kinetics of glucose permeation can then be studied independently of the kinetics of the intracellular transformation of glucose. Since this approach appears to be impracticable, the kinetics of the utilisation of glucose have been analysed on the assumption that they are determined by the kinetics of the permeation and of the phosphorylation of glucose. The results of this analysis, presented in the fourth section of this thesis, indicate that the assumption provides a fair representation of the kinetics of glucose utilisation and allows an estimate of the parameters of glucose permeation to be made with more accuracy than has been achieved hitherto.

SECTION I

AN APPARATUS FOR THE PERFUSION OF THE ISOLATED RAT HEART

Figure 2.



Principle of the Perfusion Apparatus

P = volume of recirculated perfusate.

x = concentration of metabolite in the perfusate.

i = rate of infusion and withdrawal.

a = concentration of metabolite in the infusate.

h = rate of uptake of the metabolite by the perfused organ.

CHAPTER ONE

INTRODUCTION

It was argued in the General Introduction that the study of the kinetics of glucose permeation into cardiac muscle is best conducted with an isolated perfused heart which is in a steady state of glucose permeation and utilisation. The closed circuit perfusion of a heart, where the concentration of the perfusate glucose must fall continuously, does not permit the estimation of glucose utilisation in a defined steady state. A prerequisite for this investigation was, therefore, a method for cardiac perfusion whereby the heart is established and maintained in a steady state in which the uptake of glucose may be measured. Fig. 2 illustrates the principle of a system which satisfies this requirement.

A solution of glucose is infused at a rate "i", ml./hour, and a concentration "a", mg./[redacted] ml., into a volume "P", ml., of perfusate at a glucose concentration "x", mg./[redacted]ml. The volume of perfusate is kept constant by reducing it at the same rate, "i", ml./hour. A heart, through which the perfusate is recirculated, consumes glucose at a rate "h", mg./hour. The concentration of glucose in the perfusate will tend towards the level at which it satisfies the steady state where the amount of glucose added to the perfusate equals that removed by virtue of the withdrawal of perfusate and by the utilisation of glucose by the heart. Thus:-

$$i\,a = i\,x + h$$

The utilisation of glucose at a constant concentration of perfusate glucose, x, is given by:

$$h = i\,(a - x)$$

The practical merit of an apparatus which is constructed on this principle depends upon the duration of the perfusion necessary to establish a constant concentration of perfusate glucose and the precision in the measurement of glucose utilisation which can be attained with the apparatus. The accuracy of the estimate of the difference between the glucose concentration of the infusate and perfusate will largely determine the precision with which glucose utilisation can be determined. A slow infusion rate, which will favour a large difference in concentration, will therefore be conducive to exactitude in the measurement of glucose uptake.

The time taken to establish a constant concentration of perfusate glucose is clearly dependent on the ratio of the volume of the recirculated perfusate to the infusion rate. If the rate of utilisation of glucose is assumed to be constant throughout the approach to the steady state, the rate of change in the concentration of perfusate glucose is given by:-

$$\frac{dx}{dt} = \frac{1}{P} \cdot (ia - ix - h)$$

$$\therefore \qquad \frac{dx}{dt} = \frac{i}{P} \cdot (c - x) \qquad \text{where } c = a - \frac{h}{i}$$

Rearranging and integrating:

$$-\ln(c-x) = \frac{i}{P}.t + \text{Constant}$$

When $t = 0$, $x = x_o$

$$\therefore \quad \text{Constant} = -\ln(c - x_o)$$

$$\therefore \quad \ln \frac{c - x_o}{c - x} = \frac{i}{P}.t$$

In a steady state, when $\frac{dx}{dt} = 0$ and the concentration of perfusate glucose is X:-

$$ia = iX + h$$

$$\therefore \quad X = a - \frac{h}{i} = c$$

$$\therefore \quad \ln \frac{X - x_o}{X - x} = \frac{i}{P}.t$$

At $t_{\frac{1}{2}}$ when $X - x = \frac{1}{2}(X - x_o)$

$$\ln 2 = \frac{i}{P}.t_{\frac{1}{2}}$$

$$\therefore \quad t_{\frac{1}{2}} = 0.693\ P/_i$$

The rapid attainment of a steady state is therefore favoured by a high rate of infusion, while for accurate measurements of glucose uptake a low

rate is preferable. This conflict of objectives in the application of the principle can be minimised by the use of a small volume of perfusate and by a high degree of precision in the estimation of glucose concentration.

A maximum value for the half-time of the approach to a steady state can be assessed from the limit which must be imposed on the duration of a perfusion. A heart, whose condition is deteriorating, cannot be maintained in a stable steady state and its useful perfusion is restricted to that period when the biochemical and physiological relevance of the preparation to the in vivo situation is acceptable. In the absence of any exogenous source of nutrient, a perfused heart becomes hypodynamic after 40 to 90 minutes (Fisher and Williamson, 1961a), an effect which presumably reflects the exhaustion of the endogenous reserves of glycogen and fat. When the conditions of an experiment are such that the glucose uptake is less than maximal, for example in the absence of insulin, the requirements of a perfused heart for energy may be met in part by the consumption of endogenous reserves. Indeed, in these circumstances the dependence on reserves is likely to be considerable because the maximum rate of glucose utilisation in the presence of insulin is sufficient to account for the rate of oxygen consumption of a perfused heart only if the glucose is assumed to be completely oxidised (Fisher and Williamson, 1961a). It is, therefore, possible that a heart which uses only half the maximal amount of glucose will become hypodynamic from lack of sufficient nutrient after about two hours of perfusion. Even when an exogenous substrate is sufficient to meet fully the energy requirements of a heart, techniques for cardiac perfusion are not, at present, developed to that degree of perfection where the condition of a preparation can be expected to remain

stable indefinitely. For these reasons and for the sake of comparison with the results of other workers, few of whom have extended their observations into a third hour of perfusion, the properties of an apparatus for cardiac perfusion in the steady state must enable the state to be established within two hours.

Mention was made in the General Introduction of changes in the permeability of cardiac muscle to monosaccharides during the first 30 minutes of perfusion without added insulin. Since the attainment of a steady state depends upon the initial stability of the properties of a perfused heart, the approach to a steady state is restricted, for experimental purposes, to a maximum of 90 minutes and therefore to a maximum half-time of about 12 minutes.

An upper limit can also be set to the volume of recirculated perfusate. This limit is determined by the half-time of the approach to a steady state and by the precision with which a concentration of glucose can be measured. The most unfavourable circumstance for the determination of the difference between the concentrations of glucose in the infusate and the perfusate will occur when the rate of utilisation of glucose is low at a high concentration of perfusate glucose. Bleehen and Fisher (1954) found that the maximum rate of glucose utilisation by a rat heart perfused in the absence of insulin is about 3 mg./hour which would produce a concentration difference between infusate glucose and perfusate glucose of 10 mg./100 ml. when the infusion rate is 0.5 ml./min. In the second section of this thesis, it will be shown that glucose concentrations can be determined with the standard deviation of 0.5% of the mean of quadruplicate estimations. Even at this level of

precision, a concentration difference of 10 mg./100 ml. in 200 mg./100 ml. could not be measured with an error of much less than 15%. The volume of recirculated perfusate of a system in which the half-time for the approach to a steady state is 12 minutes and the infusion rate is 0.5 ml./min. must be 8.6 ml. This upper limit for the volume of perfusate recirculated in an apparatus suitable for the study of glucose permeation in the rat heart has been derived by assuming that the least favourable conditions for the study obtain. Nevertheless, the variety of experimental conditions which can be investigated may be unacceptably limited if a satisfactory physiological state cannot be maintained in a heart which is perfused with 8 to 9 ml. of a recirculated medium.

The second chapter of this section comprises a review of the development and basic requirements of the technique of cardiac perfusion. In particular, two systems of perfusion, whose use has been predominant in the investigation of the permeation of sugars in cardiac muscle, are considered for their possible adaptation to meet the demands of the study of glucose permeation. Neither apparatus is judged to be suitable for adaptation. One system, which was developed by Bleehen and Fisher (1954) and improved by Zachariah (1961) and Gilbert (1963), requires a large volume of perfusate. The other system, which was introduced by Morgan, Henderson, Regen and Park (1961), does not appear to permit adequate control over the temperature and pressure at which a heart is perfused.

In the third chapter, a detailed description is given of a novel apparatus, which has been constructed to satisfy the requirements of a study of glucose permeation in the steady state. The animals from which hearts have been

taken, the preparation of their hearts for perfusion and the constitution of the medium with which their hearts have been perfused are the subject of the fourth chapter. Evidence is presented, in the fifth chapter, of the condition of hearts when they are perfused in an apparatus which is unattested by the experience of other users and, in the sixth chapter, the merits and limitations of the apparatus are discussed.

CHAPTER TWO

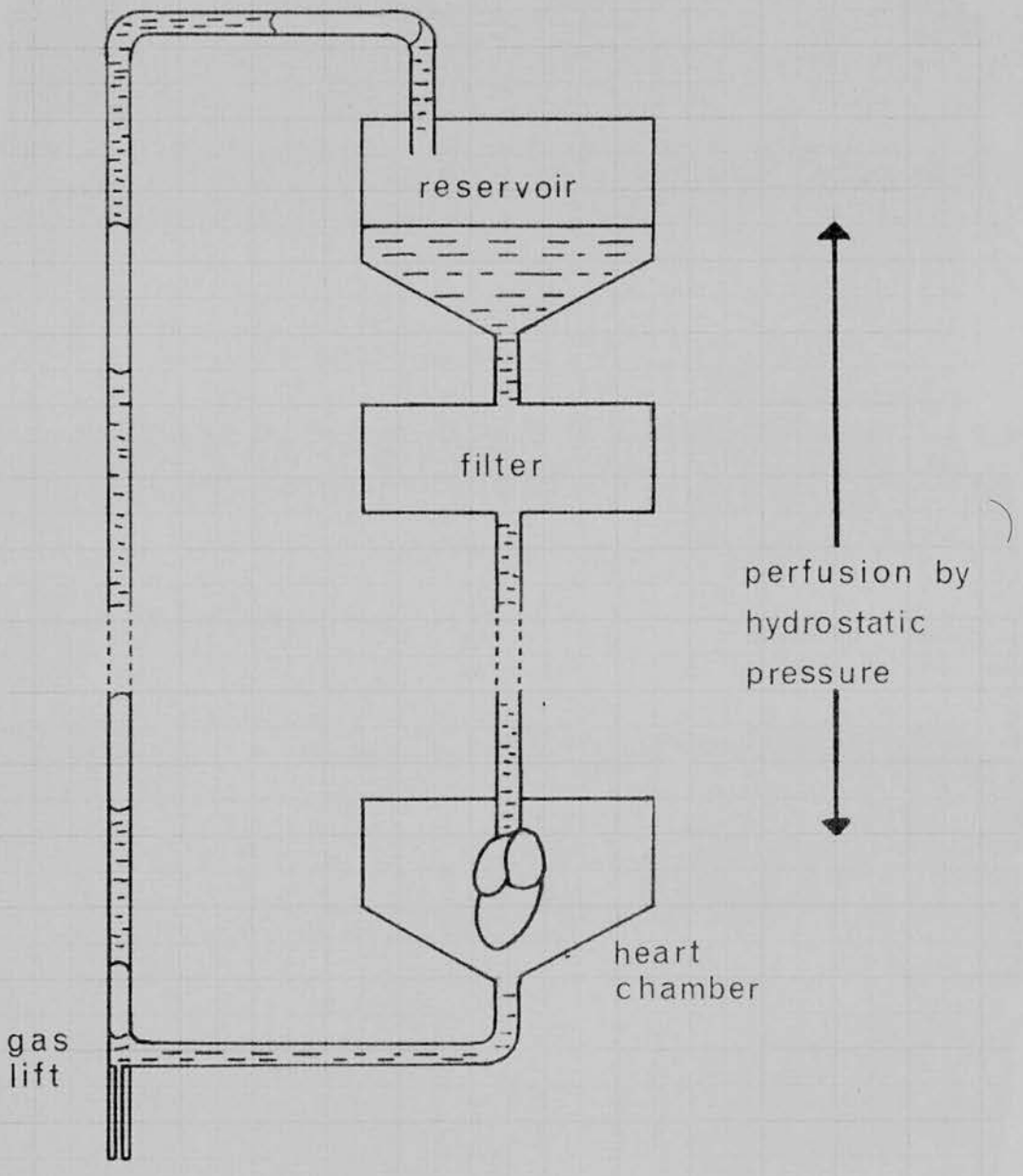
METHODS FOR THE PERFUSION OF THE MAMMALIAN HEART

AN OUTLINE OF THE HISTORY OF CARDIAC PERFUSION

The concept that an isolated organ may be usefully studied when its autolysis is prevented by artifical perfusion has been attributed to Le Gallois (1812). Despite this early inception of interest in artifical perfusion, little was contributed to an understanding of the metabolic properties of the isolated heart before the work of Langendorff, Ringer, and Locke largely laid the foundation for modern knowledge.

Langendorff in 1895 introduced the technique whereby a perfusate is supplied to a heart through the aorta. The pressure of the fluid in the aorta closes the aortic valves so that perfusate enters the coronary arteries and drains from the coronary veins into the right side of the heart. A particular advantage of the Langendorff preparation is that the heart performs no work against the external environement. The requirement of the heart for energy and, therefore, the consumption of oxygen are less in this situation than when a heart pumps perfusate or blood against a resistance. With previous methods of cardiac perfusion, which demanded that the heart perform some external work, perfusion with whole or defibrinated blood was necessary to ensure the adequate supply of oxygen to the heart. The complex and variable

Figure 3.



Principles of the Locke and Rosenheim Apparatus

constitution of blood hindered the controlled study of metabolic processes. Locke (1901) exploited the reduced requirement for oxygen of the Langendorff preparation by perfusing the isolated rabbit heart with an oxygenated saline medium which had been developed mainly by Ringer in the decade 1880 to 1890. The inclusion of glucose in the medium was found to sustain the action of the heart. Thus, at the beginning of the 20th Century the controlled investigation of carbohydrate metabolism in the isolated mammalian heart became possible.

METHODS FOR CLOSED CIRCUIT PERFUSION

The Apparatus of Locke and Rosenheim

Locke and Rosenheim (1907) proved that the consumption of glucose accompanied its beneficial effect on the isolated rabbit heart by recycling perfusate through the heart until a decrease in the concentration of the reducing sugar could be measured. The apparatus which they devised for this purpose set the pattern for the majority of the methods which have been used subsequently for cardiac perfusion when the perfusate is recirculated in a closed circuit. Fig. 3 illustrates the principle of these methods. Perfusate passes from a reservoir through a filter to the heart. The pressure at which the heart is perfused is determined by the height of the perfusate, in the reservoir, above the heart. Effluent from the heart is raised to the reservoir by a gas which is introduced under pressure at a T-piece with one capillary side-arm. Pure oxygen was used when Locke's modification of Ringer's solution was the preferred perfusate, but a mixture of 5% carbon dioxide and 95% oxygen has, in more

recent times, been used in conjunction with derivatives of the bicarbonate medium of Krebs and Henseleit (1932). In either case, the gas serves the dual purpose of oxygenation and circulation of the perfusate. In the apparatus of Locke and Rosenheim, the perfusate was filtered through glass wool and, when it was passed through a coil immersed in a water-bath, warmed to a physiological temperature before entering the heart.

This apparatus provides for all the precautions which must be taken in sustaining the activity of the isolated mammalian heart, although it is imperfect in several respects. If it may be assumed that the perfusate constitutes an adequate substitute for blood, the precautions comprise the regulation of the temperature, oxygenation and filtration of the perfusate and the control of the pressure at which the heart is perfused. The advantages in the control of the temperature, at which a metabolic or physiological process is studied, are evident. The remaining precautions together ensure that the requirements of a respiring tissue for oxygen are met, by maintaining an adequate flow of a solution of oxygen through a vascular system which does not become obstructed by debris carried in the perfusate. A supply of nutrient and the removal of waste products are also assured thereby.

Developments of the Apparatus of Locke and Rosenheim

Numerous improvements have been incorporated into the apparatus of Locke and Rosenheim without altering the principle whereby a gas-lift and hydrostatic pressure maintain the recirculation of perfusate. The majority of the components have been water jacketed for the better regulation of temperature. Devices have been elaborated to prevent the frothing of the

perfusate when it leaves the gas-lift. But it is in the manner of filtration where the greatest variety of technique has flourished.

Bleehen and Fisher (1954) replaced the glass wool filter with a Soxhlet thimble which lined the reservoir. While this modification preserved the perfused rat heart in apparently better condition than had been attained thereto the injection of crystal violet into the heart through the cannula at the end of an experiment revealed areas of the heart which were not penetrated by the dye (Zachariah, 1960). This evidence of partial blockage of the vasculature was not observed when a fine sintered glass disc was used to filter the perfusate. However a pressure of 60 cm. of water on the disc is necessary to maintain a flow of perfusate which is sufficient for the needs of the heart. A system with two reservoirs and two gas-lifts was constructed to provide the pressures for both filtration and perfusion (Zachariah, 1961). Perfusate falling from the first reservoir through the sintered glass filter is lifted to the second reservoir whence it passes through the heart before being returned to the first reservoir.

When perfusate is recycled through a sintered glass filter, the advantages gained in the efficiency of filtration are, to some extent, offset by a deleterious effect on the permeability properties of a perfused rat heart. This effect can be nullified by the inclusion of dialysed bovine serum albumin in the perfusate (Zachariah, 1961). Gilbert (1963) found a hardened filter paper - Whatman No. 50 - to be as effective a filter as sintered glass and to have no effect on the permeability of a perfused heart, but duplication of the reservoir and gas lift remained necessary. The effect of different means of filtration on the permeability of cardiac muscle will be discussed in

Figure 4.

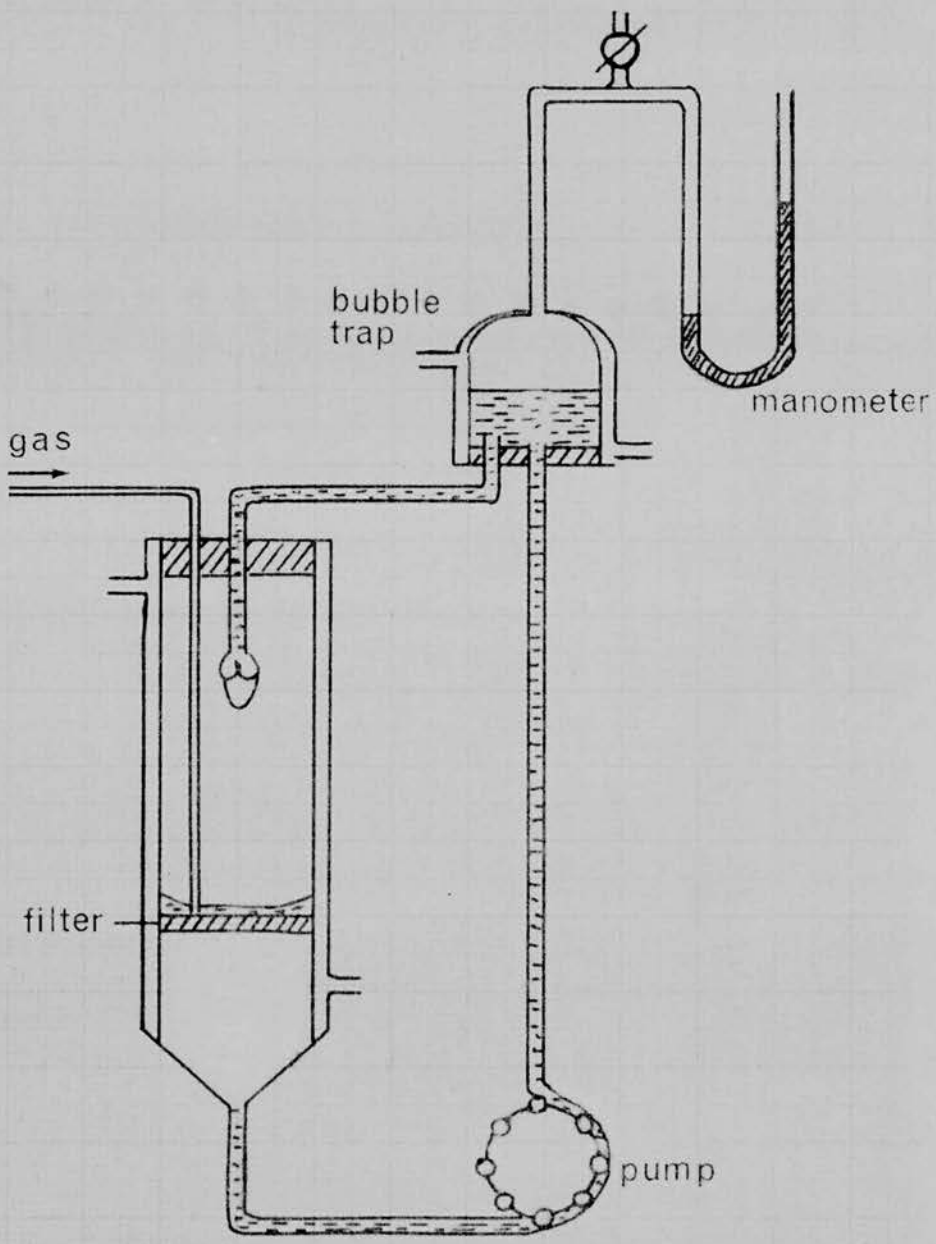


Diagram of an Apparatus for Cardiac Perfusion, described by Morgan, Henderson et alia (1961).

more detail in Section II.

A system for cardiac perfusion, which recycles perfusate on the principle of the original apparatus of Locke and Rosenheim has the merit that the control of the temperature, the pressure of perfusion, and the efficiency of the filtration and oxygenation of the perfusate are well attested. Appropriate construction and the water-jacketing of all the components will ensure that significant differences in temperature do not exist within an apparatus. The perfusion pressure is simply controlled through the height of perfusate above the heart and the efficacy of the gas-lift in maintaining the saturation of the perfusate with oxygen has been established (Fisher and Williamson, 1961b). The minimum volume of perfusate necessary to complete the circulation is about 25 ml. Since the maximum volume of the recirculated perfusate must be about 8 to 9 ml. in a practicable apparatus for the study of glucose permeation in a maintained steady state, a system which depends on hydrostatic pressure for the perfusion of a heart and a gas-lift for the completion of the re-circulation of perfusate cannot be envisaged.

An Apparatus for Closed Circuit Perfusion with a Small Volume of Perfusate

An apparatus for the perfusion of the isolated rat heart with a small volume of recirculated perfusate has been described by Morgan, Henderson, et al. (1961). In this system (Fig. 4) the perfusate is recirculated by a peristaltic pump. The heart is suspended in a water-jacketed chamber above a coarse sintered glass filter, which is sealed into the chamber. A mixture of 5% carbon dioxide and 95% oxygen is introduced into a small pool of the coronary effluent on the surface of the filter and is free to escape from

the chamber. Perfusate is drawn through the filter and returned to a water-jacketed bubble-trap by the action of the peristaltic pump. The pressure at which the heart is perfused is determined by the pressure of the gas over the perfusate in the bubble trap which is closed to the atmosphere by a mercury manometer.

This apparatus, which can be used with only 5 ml. of recirculated perfusate, satisfies with ease the requirement for a system with a maximum volume of 8 to 9 ml., but it appears not to permit adequate control over the conditions of a perfusion. The major criticism of this system concerns the provision which is made for the regulation of the temperature of the perfusate. Both the bubble-trap and the heart chamber are jacketed with water at 37°C and the gas mixture is equilibrated with water at 37°C before it is introduced into the apparatus, but the tubes which connect the components, and are about 30 cm. in their combined length, are neither water-jacketed nor insulated. When the total volume of recirculated perfusate is small, the fraction of the whole which occupies the interconnecting tubes may be substantial and the turnover of the perfusate in the chambers will certainly be rapid. The possibility that the temperature of the perfusate may vary within the system is considerable. Morgan, Henderson et al. (1961) do not refer to the temperature at which perfusate enters the heart. However, R.B. Fisher (personal communication) investigated the temperature to which a thermometer places in the heart chamber could be raised by the stream of perfusate issuing from the cannula. Superfusion of the thermometer for 10 to 15 minutes did not raise its temperature above 30 - 35°C although the environmental temperature was over 20°C. In a similar apparatus, Neely, Liebermeister, Battersby and Morgan (1967a)

observed that this temperature rose from 34.0°C to 34.8 C with a two-fold increase in the flow of perfusate through the heart.

The apparatus of Morgan, Henderson et al. may be deficient in control over the pressure as well as over the temperature of perfusion. The principle of the apparatus would appear to afford control over the pressure of perfusion by introducing gas into the bubble trap through the action of the pump or by removing gas through a tap in the connection to the manometer. Somewhat ambiguously, Morgan, Henderson et al., in describing the general characteristics of their preparation, state that the perfusion pressure of 35 to 50 mm. Hg was constant. If it is assumed that this statement refers to the variation in the perfusion pressure within a series of experiments and that the pressure was constant in any one experiment, it must be concluded that the apparatus affords poor control over the choice of a perfusion pressure at the initiation of perfusion.

Although this apparatus permits the perfusion of a heart with a small volume of recirculated perfusate, the inadequate control which it allows over both the temperature and pressure of perfusion does not recommend its use.

CHAPTER THREE

AN APPARATUS FOR CARDIAC PERFUSION IN A MAINTAINED STEADY STATE

THE DESIGN OF THE APPARATUS

An apparatus for cardiac perfusion has been constructed on a design suggested by Prof. R.B. Fisher. In this system, two peristaltic pumps provide directly the motive force for the filtration and recirculation of perfusate, for the perfusion of the heart, and for the introduction of a mixture of oxygen and carbon dioxide. Control over the pressure of perfusion is attained by allowing perfusate, which is pumped in excess of the flow through the coronary vessels, to by-pass the heart through a resistance which ensures that perfusate enters both the by-pass and the heart at the same pressure. Provision is also made for the infusion of perfusate into the circulation and for the withdrawal of perfusate at an equal rate.

Fig. 5 illustrates the course taken by perfusate and gas through the apparatus and the places at which perfusate is infused and withdrawn. Perfusate, which is drawn from a reservoir, is driven by the peristaltic pump through a filter whence it can proceed through either a resistance or a heart. A second peristaltic pump, on the same drive shaft, collects the effluent from the heart. The capacity of this pump considerably exceeds the rate of coronary perfusion so that the pump is also able to draw a mixture of

Figure 5.

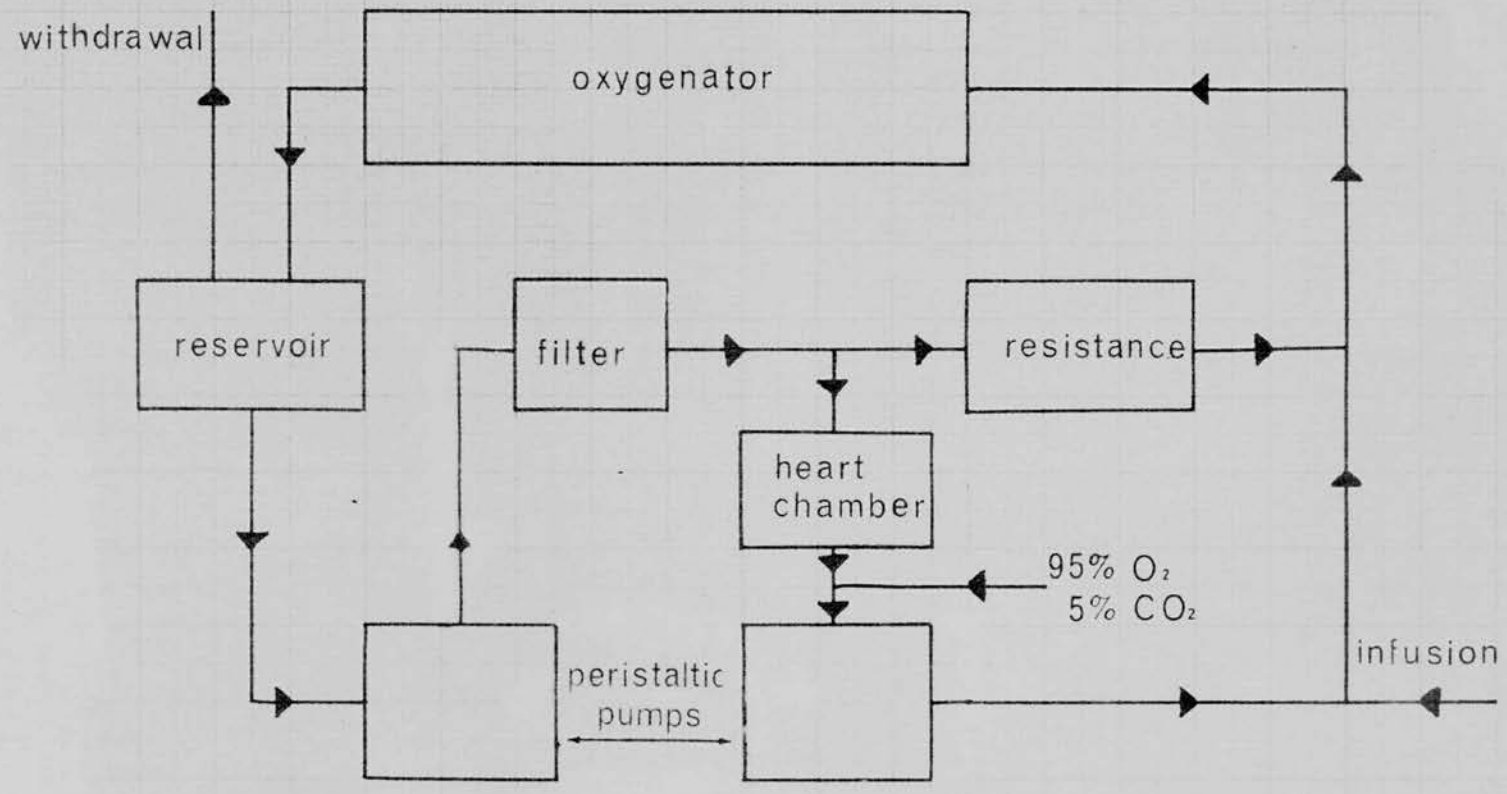
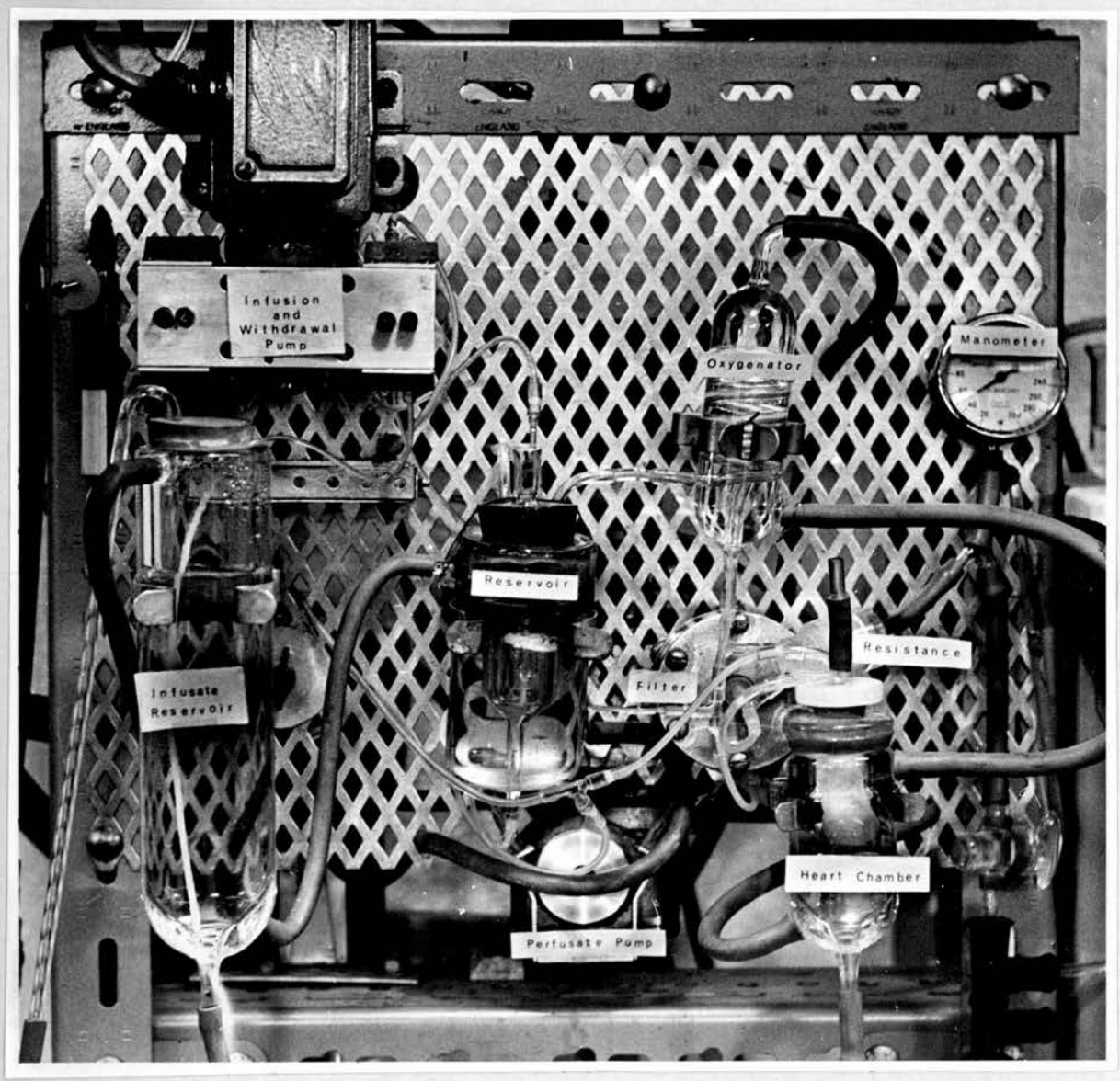


Diagram of Flow in the Perfusion Apparatus

Fig. 6



The Apparatus For Cardiac Perfusion

5% carbon dioxide and 95% oxygen into the effluent. The segmented stream of perfusate and gas, which leaves the pump, receives infusate, from a third pump, not shown in Fig. 5, and the perfusate which by-passes the heart. The whole returns to the reservoir but is delayed by passage through a coil (oxygenator) to allow time for gaseous exchange. The gas, which separates from perfusate, is free to leave the reservoir, from which perfusate is also withdrawn by a fourth pump, on the same drive shaft as the infusion pump, at a rate equivalent to that of infusion. The true relationship of the components of the apparatus is shown photographically in Fig. 6.

THE COMPONENTS OF THE APPARATUS

The Peristaltic Pumps

The peristaltic pumps used in the apparatus were constructed in the Dept. of Biochemistry by Mr. A. Purdie and Mr. W. Tait. A design, which is the result of a process of evolution, will be described but earlier products of the development have been used in this investigation. Modifications were made in the pumps only for the sake of experimental convenience and they have not altered the characteristics of the system of perfusion.

The pumps which circulate and introduce oxygen into the perfusate, are driven by a series universal Parvalux motor, Type SD6, which is controlled by a variable transformer. The pumps which are responsible for the infusion and withdrawal of perfusate, are driven by a D.C. shunt Parvalux motor, Type SD6, which is linked to a Sanders solid state control unit (W.H. Sanders (Electronics) Ltd. Herts.). This form of control, which is operated by feed-

Figure 7.

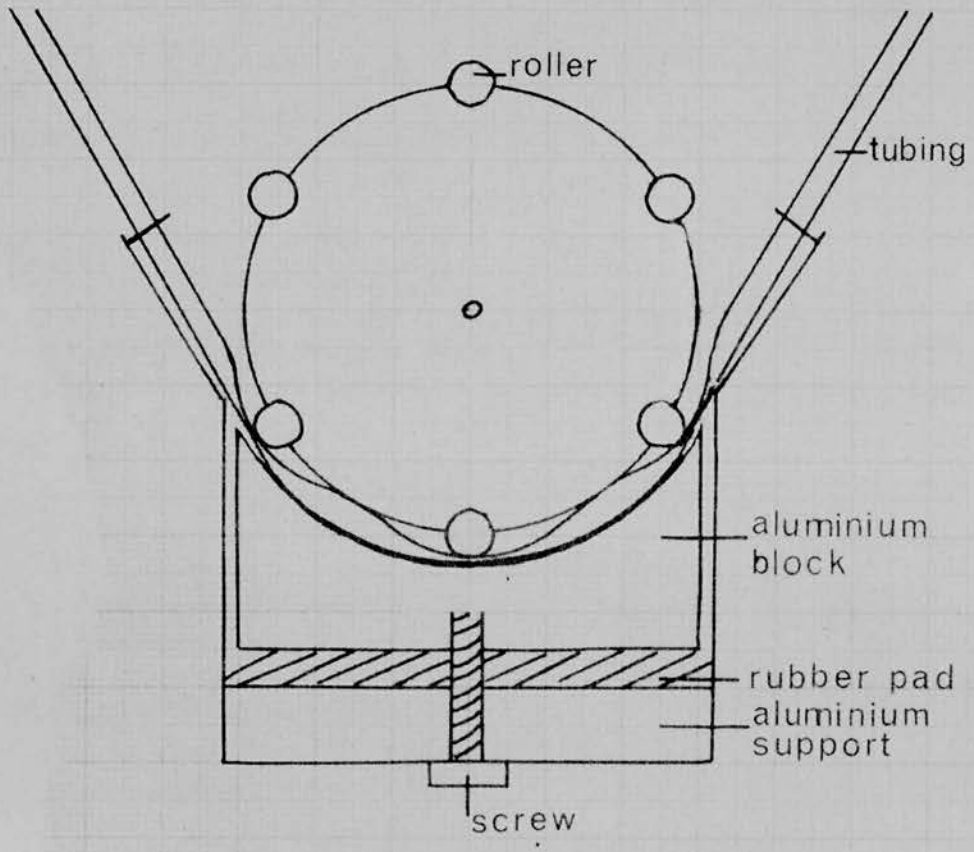
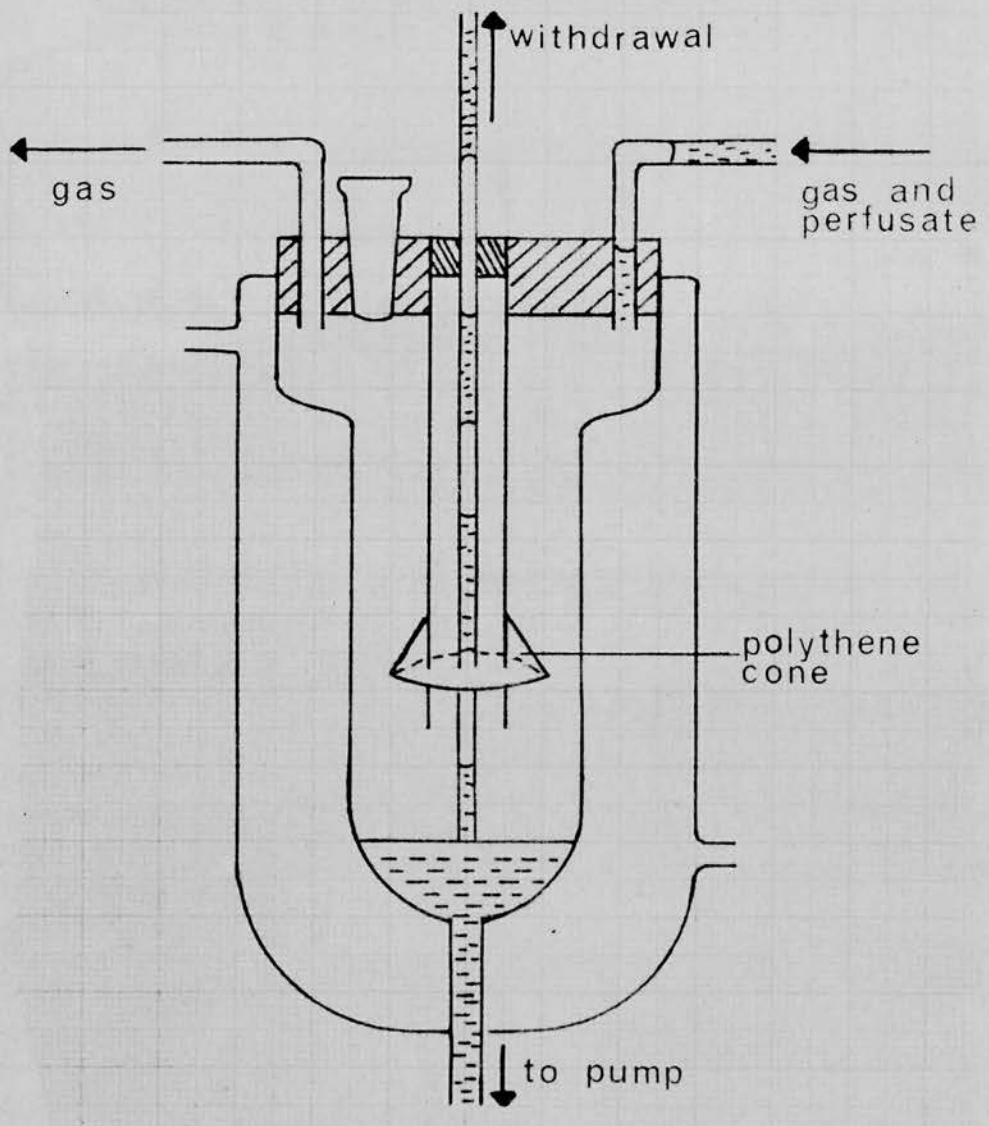


Diagram of Pump Unit [not to scale]

Figure 8.



Perfusate Reservoir

back from the output of a tachogenerator driven by the motor, ensures a constant rate of infusion but allows the rate to be varied continuously over a wide range as desired.

The construction of the Units which transmit the drive of the motors to the contents of Tygon tubing is identical for both motors, Fig. 7. Two aluminium discs (diameter 1.5 in.) are mounted through their centres on an axle of the motor. Between them, the discs support six brass rollers (diameter 0.25 in.) at equidistant intervals about their circumferences. Each roller is free to rotate and can compress tubing between it and an aluminium block, whose surface is machined to form an arc of the circle described by the rollers so that the tubing is always compressed by at least two and at the most three of the rollers. The clearance between the rollers and the aluminium block can be varied to permit the use of the entire range of Tygon tubing which is supplied by Technicon Instruments Ltd. This facility is achieved by placing a pad of rubber between the aluminium block and a rigid support which is fixed to the motor itself. A screw, which passes through the support and the rubber pad from the aluminium block, enables the block to be drawn down on the pad, which is, however, sufficiently firm to resist distortion by the action of the rollers. Several independently adjustable units, which consist of the brass rollers, the aluminium block and the rubber pad, can be mounted on a single motor. Each unit has been treated as a separate peristaltic pump.

The Perfusate Reservoir (Fig. 8)

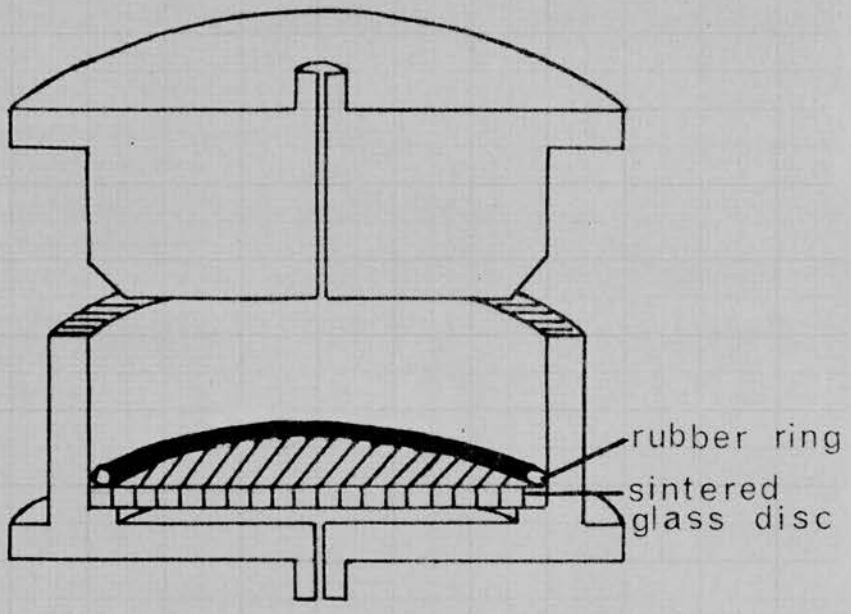
The perfusate reservoir is water-jacketed. It has a wide mouth (3.0 cm. diameter) closed by a rubber bung through which pass tubes which permit the

return of the mixture of gas and perfusate from the rest of the system, the exit of the gas from this mixture and the withdrawal of perfusate from the circulation. The bung also holds a stoppered Quickfit neck through which the perfusate is initially introduced into the apparatus. The main section of the reservoir has a diameter of 2.0 cm., but there is a space below the bung sufficient to allow the gas to separate from the perfusate without the uncontrollable formation of froth. The reservoir tapers to a tube (diameter 2.0 mm.) through which perfusate is drawn to a peristaltic pump.

A constant volume of perfusate in the apparatus is achieved by maintaining the perfusate at a fixed level in the reservoir. A length of quill tubing, tipped with a piece of bile duct tubing, which serves to reduce the surface interaction with the perfusate, passes through the middle of the bung and reaches almost to the bottom of the reservoir. A peristaltic pump with a greater capacity than that which infuses perfusate into the system is connected to the quill tubing. Gas and perfusate are withdrawn through the tube and the level of the perfusate in the reservoir is maintained at the tip of the bile duct tubing. Perfusate is necessarily withdrawn from the reservoir at a rate which equals that of the infusion of perfusate into the circulation.

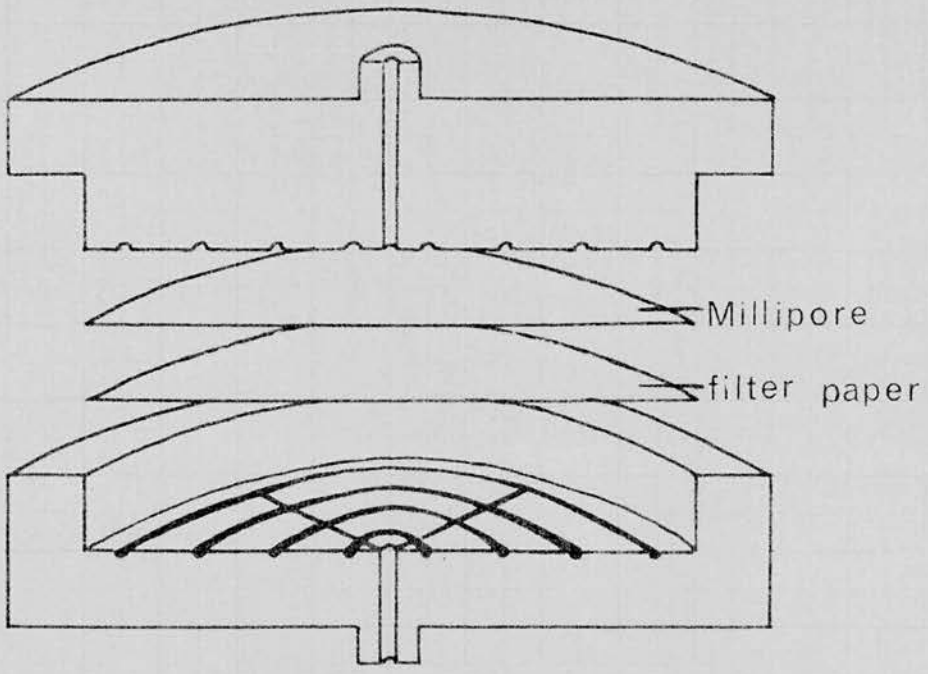
The quill tubing is introduced into the reservoir through a tube of larger diameter which reaches to within two cms. of the surface of the perfusate and supports there a polythene cone which extends almost to the walls of the reservoir. This device prevents perfusate from flowing down the outside of the quill tubing and being then pumped from the reservoir, with consequent fluctuations in the level of the perfusate. The cone is also effective in dispersing any residual froth, thereby ensuring an undisturbed

Figure 9.



Sintered Glass Filter

Figure 10.



Millipore Filter

meniscus.

The Filter

Perfusate, pumped from the reservoir, is filtered before it enters any other part of the apparatus. In this work, both sintered glass and Millipore filters have been used.

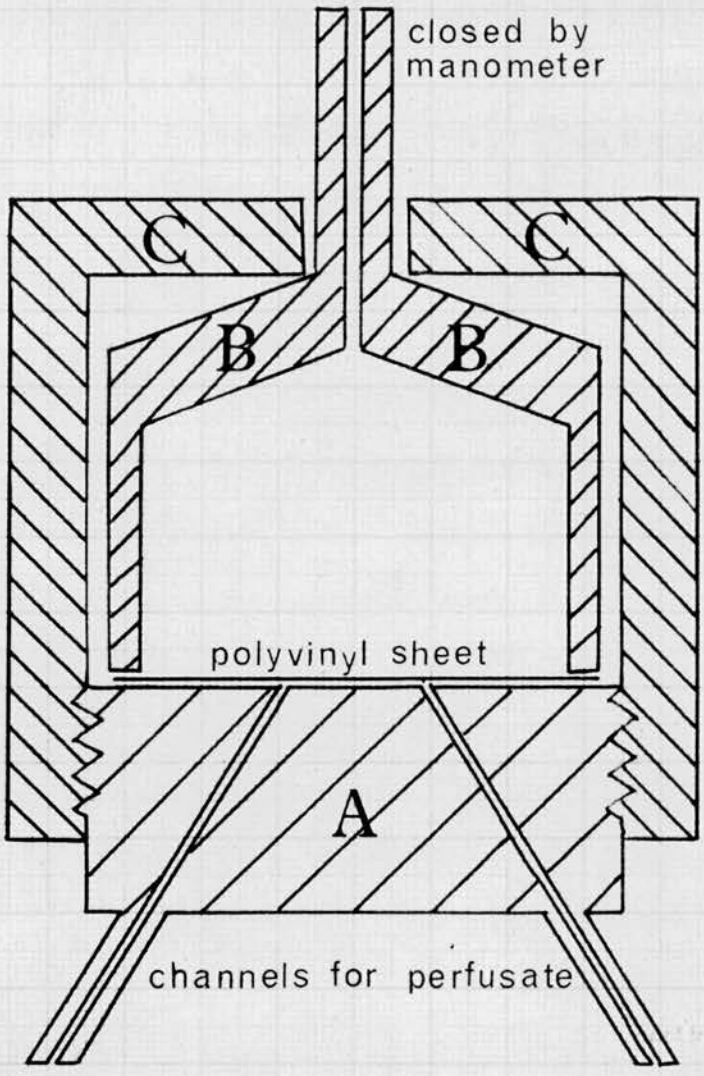
a) Sintered Glass Filter (Fig. 9)

A Pyrex sintered disc (porosity 3, diameter 20 mm) is supported within a polythene block above a depression, 1 mm. deep. A second polythene block, which is machined to make a close fit with the first, holds the filter in place when the two blocks are clamped together. The load of the second block is distributed around the supported edge of the disc by a rubber ring which makes a water-tight seal between the disc and the polythene. The ring also allows perfusate to cover the maximum possible area of the filter by preventing direct contact between the male block and the sintered disc. Perfusate has access to the filter through axial channels (diameter 1.5 mm.) in the two blocks and is introduced through the male component. The design allows the efficient use of the sintered disc while it reduces to a minimum the volume of perfusate which is held by the whole assembly.

b) Millipore Filter (Fig. 10)

Two circular Perspex blocks are machined, one to have a depression 0.5 cm. in depth and 4.7 cm. in diameter, and the other to have an equivalent interlocking projection. The face of each block is milled in an identical pattern of ten concentric channels and four radial channels, which are at right angles and intersect in a central hole (diameter 1.5 mm.) bored through the block.

Figure 11.



Resistance

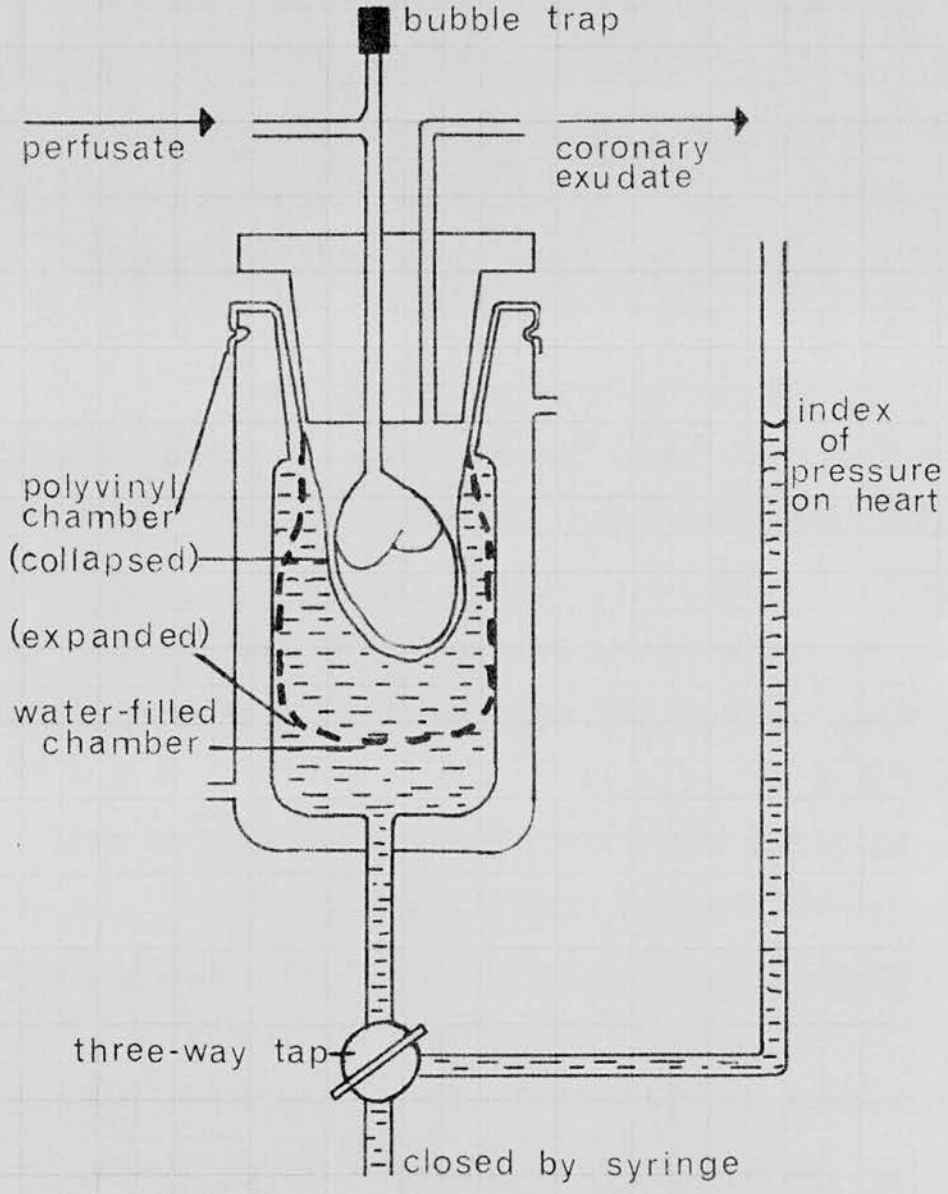
The channels are 0.5 mm. in depth. A disc of hardened filter paper (Whatman No. 50) lines the depression and supports a disc of Millipore (pore size, 3μ; diameter 4.7 cm.). A rubber ring makes a seal when the two Perspex blocks are bolted together through aluminium plates which back each block. Perfusate, which is introduced through the projecting block, is distributed in the channels over the surface of the Millipore filter and collected in the opposed set of channels. Again, the design allows efficient filtration without a large volume of perfusate being necessary to fill the device.

A Resistance Regulated by Air Pressure (Fig. 11)

Perfusate which issues from the filter can either by-pass the heart through a resistance or perfuse the heart at the pressure determined by the resistance. The resistance operates essentially on the principle of the "Starling resistance" (Knowlton and Starling, 1912) in which the pressure exerted by air on a collapsible rubber tube determines the pressure of the liquid which enters the tube.

In the device constructed for this work, perfusate enters and leaves through channels (diameter 1.5 mm.) which pass through a cylindrical block (A) of Perspex (diameter 1.9 cm.) and open on the circular surface with a distance of 8 mm. between their centres. The channels are not parallel but converge to make an angle of 22°. This convergence of the channels reduces the inherent resistance of the device which limits the range over which control can be exerted on the pressure of perfusion. Perfusate passes from one channel to the other under a thin circular polyvinyl sheet, whose edges are held firmly on the surface of A by a partly hollow cylinder of Perspex (B),

Figure 12.



Heart Chamber

which, in turn, is kept in place by a third piece of Perspex (C). This last piece of Perspex is also a partly hollow cylinder which fits over B and can be screwed on to A. The pressure of the air over the polyvinyl sheet can be varied by the introduction or the release of air through b, which protrudes through C. An Accoson sphygmomanometer measures the pressure of the air. When the passage of the perfusate under the polyvinyl sheet constitutes the greatest resistance in the by-pass to the flow of perfusate, the pressure over the sheet determines the pressure at which perfusate enters the by-pass and the heart. The efficacy of this device will be discussed when the properties of the perfusion apparatus are described.

The design of the by-pass is the result of an evolution, as was the case for the peristaltic pumps. Other versions, which have been used in this work and were constructed on the same principle, contained a sheet of rubber, which was cut either from a toy balloon or a Durex contraceptive, stretched over a bung. These versions were superseded because polyvinyl, unlike rubber, is not attacked by the solution of sodium hypochlorite with which the apparatus is cleaned and because the properties of the device are reproducible when the sheet, whether of rubber or polyvinyl, is not stretched during the construction of the by-pass. Whatever the manner of the construction of the by-pass, all experiments on the rate of glucose uptake in the steady state have been conducted at the same perfusion pressure, 60 cm. of water.

The Heart Chamber (Fig. 12)

The heart chamber consists of an outer water-jacketing compartment and an inner compartment which is filled with water, capped by a polyvinyl finger

and connected, at the bottom, to a syringe. The polyvinyl finger, which is cut from a "Wilson Tru-touch ambi" glove (manufactured by Becton, Dickinson & Co., Canada, Ltd.) is moulded to the shape of the mouth of the chamber by stretching it over an aluminium former and heating it at 100°C for 10 minutes. (In the early stages of this investigation, a Durex contraceptive was used in place of the polyvinyl finger). When water is drawn from the inner compartment into the syringe, the finger expands within the chamber and allows a heart, which is mounted on a cannula held in a polythene bung, to be introduced into the finger. With the bung in place, the finger can be collapsed gently around the heart by returning water from the syringe. To ensure that a large pressure is not imposed upon the heart, a three-way tap between the chamber and the syringe allows the water in the inner chamber to be made continuous with a column of water which rises to not more than 2 or 3 cm. above the heart. The effluent from the heart leaves the finger through a tube in the polythene bung, so that the heart is immersed in perfusate which ensures good thermal contact with the water-jacketing. In an apparatus which is designed to allow the perfusion of a heart with a small volume of recirculated perfusate, the use of a collapsible heart chamber is particularly advantageous because about 5 ml. of perfusate would be necessary to immerse a heart in a rigid chamber.

The Oxygenator

A gas mixture of 5% carbon dioxide and 95% oxygen is drawn into the apparatus by the peristaltic pump which also receives the coronary effluent. Gaseous exchange can therefore occur from the time of the union of perfusate and gas at a T-piece from which the mixture is taken into the pump. The

output from the pump receives infusate and the perfusate which has by-passed the heart. The whole passes through a water-jacketed coil whose function is to create a delay of about 5 seconds in the return of the perfusate to the reservoir. This coil will be referred to as the oxygenator although oxygenation can occur wherever the perfusate is exposed to the gas mixture.

The Infusate Reservoir

Infusate, which is to be pumped into the apparatus, is stored in a water-jacketed vessel. The contents of the reservoir are recycled and oxygenated in a gas lift by the gas mixture of 5% carbon dioxide and 95% oxygen. Infusate returns to the reservoir through a tube in a bung which largely closes the mouth of the reservoir. A groove in the bung allows the escape of gas from the gas-lift and access to the reservoir for Tygon tubing linked to a peristaltic pump.

Interconnections and Junctions

All interconnections between components of the apparatus are made with Tygon tubing and all junctions are glass T-pieces. The length of the interconnections is reduced to a minimum, but it has not been found necessary to insulate the tubing. Where practicable, narrow bore tubing is used to reduce the volume of perfusate contained in these unjacketed regions.

THE CHARACTERISTICS OF THE APPARATUS

The volume of Perfusate

An accurate estimate of the volume of recirculated perfusate is not readily obtained. Attempts to empty the apparatus at the end of a perfusion did not succeed in completely exhausting the system and yielded 5.0 to 5.5 ml. of perfusate. Determination of a volume of water, which was circulated in the absence of a heart, from the dilution of a known amount of glucose yields values of 5.5 to 6.5 ml. However, the circumstances do not mimic precisely those which obtain during the perfusion of a heart. Unless it can be made in each experiment, the precise estimation of the volume of recirculated perfusate is of little value because the impossibility of reproducibly collapsing the polyvinyl finger round each heart results in variation of the volume of perfusate between experiments.

The estimation of glucose utilisation in a steady state does not require any knowledge of the volume of perfusate, but this knowledge is necessary when the time-course of glucose utilisation is determined. For this latter purpose, the volume of perfusate has been taken to be 6.0 ml. The contribution of the error in this figure to the accuracy of the whole computation will be discussed in Section II. It is clear, however, that the volume of recirculated perfusate is comfortably below the upper limit of 8 to 9 ml. for a practicable system.

The Rate of Infusion

For the majority of the work reported here, the infusion rate has been

approximately 0.5 ml./min. The rate has been measured in two ways. The time in which the infusion pump aspirates a known volume of perfusate has been determined before and after a perfusion. When this process is continued through a series of successive experiments, the infusion rate is found to remain constant for at least 6 hours. For an infusion rate of 0.5 ml./min. which is measured in this manner, the standard deviation is estimated to be 0.005 ml./min. Alternatively, the infusion rate can be measured by weighing the perfusate which is withdrawn from the apparatus over an interval of 4 minutes. This procedure is convenient, because the same process yields samples of the perfusate for glucose estimation, but it is the less accurate of the two. For an infusion rate of 0.5 ml./min. which is measured by the second method, the standard deviation is estimated to be 0.009 ml./min. Although the mean values of an infusion rate determined by both methods are insignificantly different, the greater standard deviation of the value obtained by the second method presumably reflects the fact that the aspiration of perfusate from the reservoir is never continuous but is characterised by intervals of 4 to 5 seconds in every 15 to 20 seconds when gas alone is pumped. The method of measurement by the aspiration of a known volume of perfusate was adopted in the later stages of this investigation and the results which will be presented were obtained by both methods and in roughly equal proportions.

For a system with a volume of recirculated perfusate of 6.0 ml. and an infusion rate of 0.5 ml./min., the half-time of the approach to a steady state is 8.3 minutes. If a heart was initially in a stable metabolic state, perfusion for one hour would bring the system to within 1% of a steady state.

The accuracy of the determination of the difference in the concentration between infusate glucose and perfusate glucose which is established in a system when the infusion rate is about 0.5 ml./min. will be discussed in Section II.

The Output of the Perfusate Pumps

It is evident that, to attain control over the perfusion pressure, perfusate must be pumped from the reservoir at a rate greater than that which might be expected for the coronary flow. At a perfusion pressure of 60 cm. of water, the coronary flow rate is approximately 9.0 ml./min. at the start of a perfusion and falls to about 6.0 ml./min. over 90 minutes (Zachariah, 1960; Gilbert, 1963). The decrease in rate occurs mainly in the first 15 minutes of perfusion and has been attributed to the disappearance of adrenaline which was released when the animal was anaesthetised (Fisher and Williamson, 1961). Similar values for the coronary flow have been measured in this apparatus and are reported in Chapter Five of this Section. Perfusate has been pumped from the reservoir at between 15 ml./min. and 18 ml./min. so that except at the initiation of perfusion almost two-thirds of the output of the pump will by-pass the heart. An advantage of this apparatus is that the perfusate is filtered and oxygenated whether it perfuses the heart or not. Thus at an output from the pump of 18 ml./min. the flow through the filter and the oxygenator is about three times greater than the flow through the heart.

The output of the pump, which introduces the gas mixture into the apparatus and returns the coronary effluent to the reservoir, is about

50 ml./min. It therefore pumps approximately 6 ml. of perfusate and 44 ml. of gas per minute. The convergence of the two streams in a T-piece results in a high degree of segmentation of the perfusate and a large surface area of interaction between the two phases. A similar effect is produced when infusate and the perfusate which by-passes the heart join the flow. Under these circumstances, the volume ratio of gas to perfusate in the mixture which passes through the oxygenator is approximately 2 to 1.

The gas which is drawn into the apparatus has passed through a buffer bottle in which it is in contact with water at room temperature. Some water can therefore be lost from the system when it saturates the gas at 37°C. If a similar effect on the volume of the perfusate in the infusate reservoir is taken into account, the loss of water is less than 0.2% of the volume of perfusate which is circulated and infused in any period. This loss is too small to affect significantly the osmolarity of the perfusate.

The Regulation of Temperature

The water which circulates through the jacketed components of the apparatus is heated and pumped by a Circotherm (Obtained from Shandon Scientific Company Ltd.). The temperature of the water in the jackets is adjusted so that the perfusate, which enters the cannula of the heart, is at 37.4°C. Since the temperature in the bath, from which the water is pumped, is then 37.8°C, the maximum difference in temperature which could exist between the surface and the interior of the heart is 0.4°C.

The Regulation of the Perfusion Pressure

The by-pass, which has been described, does not allow the perfusion pressure to be controlled below approximately 35 cm. of water which constitutes the inherent resistance to the flow of perfusate through the device when no pressure greater than atmospheric is applied to the polyvinyl sheet. The correlation between the pressure imposed on the polyvinyl sheet and the height of a column of water at the end of the connection to the heart chamber has been examined. Provided that the bore of the tubing which connects the two is not so narrow that there is significant resistance to the flow of perfusate, the correlation is good. In these experiments, the pressure in the by-pass has been set to produce a pressure of 60 cm. of water at the entrance to the heart chamber. The perfusion pressure tends to rise slowly during an experiment. After an hour, the rise in pressure becomes negligible or ceases. The most reasonable explanation of the increase in pressure would be the gradual warming of the air in the by-pass and the connection to the sphygmomanometer. By releasing air from the by-pass the pressure is regulated so that the maximum variation in any experiment is 6 cm. of water.

CHAPTER FOUR

MATERIALS AND PROCEDURE FOR PERFUSION

ANIMALS

Hearts were taken from male Wistar rats which weighed between 170 g. and 250 g. and had had free access to food and water. The animals were kept in conditions of constant temperature and day length (12 hours) for at least two weeks before use. These precautions were taken to preclude seasonal variations in the sensitivity of the rat heart which have been observed by Young (1965).

SALINE SOLUTIONS AND THE PERFUSION MEDIUM

All the chemicals, which were used in this work, were, unless it is stated otherwise, of Analar quality and obtained from British Drug Houses Ltd. Doubly distilled water was always used for the saline solutions and the perfusion medium.

Saline Solution A

The solution contains sodium chloride, 142.2 mM/l and sodium hydrogen carbonate, 0.5 mM/l, and has therefore 285.4 milliosmoles/l. When the

TABLE 2

THE COMPOSITION OF THE PERFUSATE

Component	Concentration mM	Osmotic coefficient	Active osmolarity m. Osmoles	Solids mg./ml.
NaCl	118.480	0.93	220.373	6.93
$NaHCO_3$	24.876	0.96	47.762	2.09
KCl	4.739	0.92	8.720	0.35
KH_2PO_4	1.186	0.87	2.064	0.16
$CaCl_2 . 6H_2O$	1.270	0.86	3.277	0.28
$MgSO_4 . 7H_2O$	0.593	0.58	0.688	0.15
		Total =	282.884	Total = 9.96

Inulin, at 20 mg./ml., would increase the active osmolarity by about 3.33 m. Osmoles and raffinose at 10 mg./ml. by 19.9 m. Osmoles. In most experiments, glucose increased the osmolarity by less than 5.0 m. Osmoles and rarely by more than 10 m. Osmoles.

solution is equilibrated at 0°C with atmospheric carbon dioxide, it has a pH of 7.4.

Saline Solution B

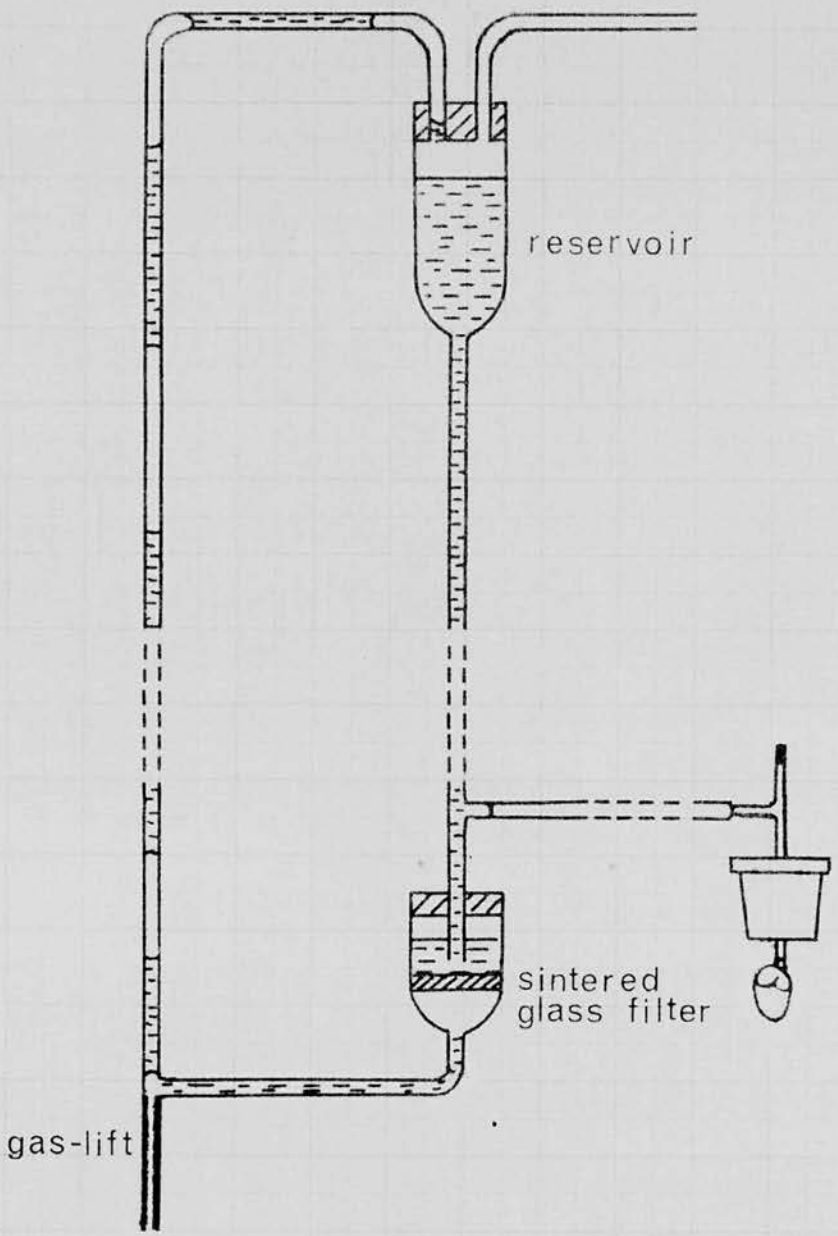
The solution contains sodium chloride, 104.28 mM/l, and sodium hydrogen carbonate, 39.8 mM/l and has therefore 288.2 milliosmoles/l. When the solution is equilibrated at 20°C and at atmospheric pressure with a gas mixture which contains 5% carbon dioxide, it has a pH of 7.4.

Perfusion Medium

The concentration and osmolarity of the electrolytes which are included in the perfusion medium are given in Table 2, where the influence of inulin or raffinose on the osmolarity of the medium is also indicated. In very few instances has a concentration of glucose greater than 10 milliosmolar been used in the perfusate. Fresh perfusate was prepared when required from stock solutions stored at 4°C. When the medium is equilibrated at 37°C and at atmospheric pressure with a gas mixture which contains 5% carbon dioxide, it has a pH of 7.4.

This medium is similar to the bicarbonate medium of Krebs and Henseleit (1932) but the concentrations of the calcium and magnesium ions are halved to allow for the binding of these ions by plasma proteins (Greene and Power, 1931).

Figure 13.



Wash-out Apparatus

THE EXPERIMENTAL PROCEDURE

Ether was used to induce anaesthesia in the rat. The thoracic cage of the rat was then opened and the heart was rapidly excised in such a way that part of the aortic arch remained attached. The heart was transferred to a dish of Saline A and agitated to remove blood. The saline was cooled (4°C) to reduce the possibility that blood might clot in the vessels of the heart and to diminish the metabolic activity of the heart during this brief period of anoxia. In a second dish of cold Saline A, where visibility was not reduced by blood, the aorta was prepared for cannulation and the majority of the adherent connective and adipose tissue was removed. The aorta was then slipped over the tip of the cannula while a slow drip of oxygenated and filtered Saline B was maintained through the cannula from an apparatus which is illustrated in Fig. 13. Linen thread was used to bind the aorta to the cannula and the flow of saline to the heart was increased by fully releasing a screw clip. The object of this procedure is to wash all the blood from the vessels of the heart with a saline solution which has been recycled for at least 30 minutes through a fine sintered glass filter by way of a gas-lift which employs a mixture of 5% carbon dioxide and 95% oxygen. The saline B, which washed the blood from the heart, was at room temperature and the heart resumed contraction at a slow rate as soon as the saline was allowed to perfuse the heart at a pressure of 60 cm. of water. Within 1 minute, the blood was washed from the heart. Any final cleaning away of tissue from the heart was then performed. The polythene bung which bears the cannula and the heart was then transferred to the main perfusion apparatus.

At this stage approximately 20 ml. of perfusate had been warmed, filtered and oxygenated by recirculation in the perfusion apparatus through the by-pass, with the leads to and from the heart chamber closed by screw clips, the polyvinyl finger of the heart chamber had been expanded and the air pressure in the by-pass had been set. The heart was connected to the lead to the heart chamber and the screw clip, which had just previously been opened to allow a slow drip of perfusate from the lead, was removed. Before the heart was inserted into the heart chamber, the coronary effluent was allowed to run to waste for approximately 10 seconds to displace the greater part of the Saline B. When the bung and the heart were in place in the heart chamber, the lead which carries the coronary effluent from the chamber was connected and the polyvinyl finger was collapsed around the heart. The pressure exerted on the heart was set to be no greater than 3 cm. of water. Infusion and withdrawal of perfusate was then started. The volume of the circulated perfusate fell progressively because the withdrawal rate is greater than the infusion rate and reached a minimum within 5 minutes of the connection of the heart to the apparatus. The time taken from the dissection of the animal to the connection of the heart to the main perfusion system was approximately 6 minutes.

In the course of a perfusion, there was no need to interfere with the system other than to make occasional adjustments to the pressure in the by-pass or to disrupt any excessive froth in the reservoir by touching the walls of the reservoir with the tip of a glass rod which was lightly smeared with Silicone Antifoam A emulsion (obtained from Hopkins and Williams Ltd.).

CHAPTER FIVE

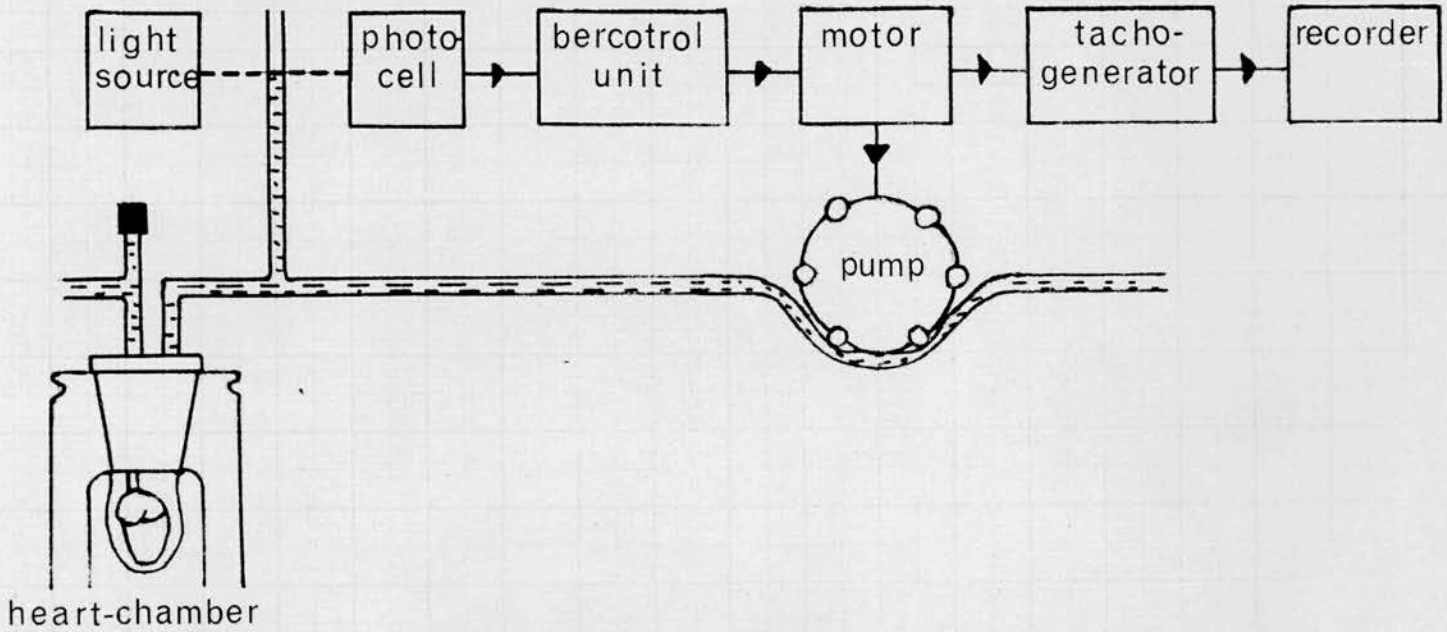
THE GENERAL CHARACTERISTICS OF THE PREPARATION

The novel apparatus which has been described differs from other systems for cardiac perfusion by the method of Langendorff in the means by which an oxygenated, warmed and filtered perfusate is presented at a controlled pressure to an isolated rat heart. If these different systems are effective in sustaining the activity of a perfused heart, the characteristics of the preparations should be comparable.

FREQUENCY OF HEART BEAT

A disadvantage of the polyvinyl finger is that it is imperfectly transparent and hinders the detection of oedema or the partial blockage of the coronary vessels during the course of an experiment. The finger does not, however, prevent the counting of the contractions of the heart which fall to 200 to 240 / min. after a period of more rapid and irregular contraction in the first five minutes of perfusion. The initial rapidity is probably due to the effects of endogenous adrenaline and the initial irregularity of contraction seems likely to be due to lack of uniformity in the warming up of the heart by the perfusate. The range of the steady rate of contraction is in good accord with the observations of Morgan, Henderson et al. (1961) and Gilbert (1963). After the period of irregular contraction a constant rate of contraction was usually maintained throughout an experiment lasting

Figure 14.



System for the Measurement of Coronary Flow

90 minutes. Variations of more than 10 contractions/min. during the course of an experiment were rare.

The vigour of the contractions could not be assessed in absolute terms, since the motion of the heart in the chamber depends considerably on the length of aorta retained and the way in which the polyvinyl finger collapses round the heart. But it is easy to make subjective judgements of comparative vigour by regular inspection throughout an experiment, and such inspections showed that it was unusual for any obvious decline to occur during 90 minutes of perfusion. When a decline in vigour did occur, it was, almost without exception, late in the perfusion of a heart without insulin. Hearts have been perfused with insulin for three hours without an apparent change in the force of contraction. Thereafter a gradual diminution in vigour was usual, but this was sufficiently slow for a heart to maintain a weak regular beat after seven hours of perfusion.

THE EFFECT OF THE PERFUSION PRESSURE ON CORONARY FLOW

The apparatus, in the form which has been described, does not allow the flow through the coronary vessels to be measured by one of the simpler methods, for example by the counting of the drips from the heart or by the timing of the passage of an injected bubble along a tube of known dimensions. In a modification of the apparatus, the coronary flow rate was measured by a method which was suggested by Prof. R.B. Fisher.

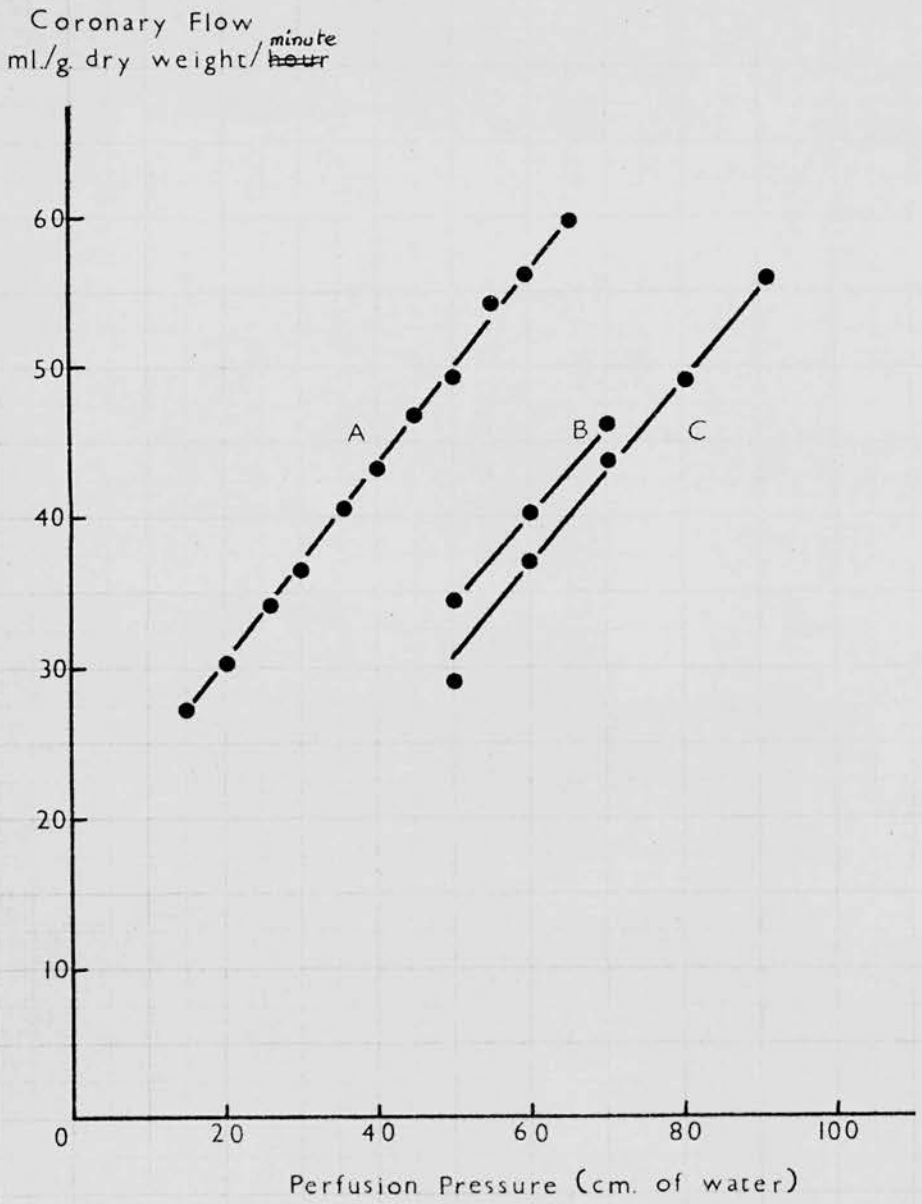
The adjunct to the perfusion apparatus is illustrated in Fig. 14. The coronary effluent enters a T-piece, whose side-arm is open and is mounted

vertically between a light-source and a photo-cell, and may either rise up the side-arm or be removed from the T-piece by a peristaltic pump. As the perfusate interrupts the light-path, the diminished output of the photo-cell, which is linked to the pump through a Bercotrol control unit (obtained from British Electric Resistance Co. Ltd., Middlesex), results in a proportional increase in the rate of the pump. In the ideal situation, perfusate would be pumped from the T-piece at the same rate as that at which it entered. The level of perfusate in the side-arm of the T-piece would then remain constant. This ideal could not be realised, instead the level of the perfusate and the rate of the pump oscillated. However, the oscillation was usually within sufficiently narrow limits to allow the estimation of the average output of the pump. The voltage generated by a tachogenerator, which was driven by the motor of the pump, was recorded on a Bausch and Lomb V.O.M-5 recorder and was used to calibrate the capacity of the pump. Although the "noise" on the record was often excessive, it was possible to perform a few experiments in which the coronary flow was measured continuously and the effect of the perfusion pressure on the coronary flow rate was investigated.

When the vagaries of the method allowed the coronary flow rate to be measured with adequate accuracy from the initiation of perfusion, the rate decreased during the early period of perfusion at a constant pressure. In the three experiments in which this observation was possible the coronary flow rate became constant after 5,20 and 30 minutes of perfusion, and a fall in rate of 31%, 24% and 43% respectively (mean 33%). After the initial decline, the coronary flow rate remained constant for up to 2 hours of perfusion at a constant pressure. When the perfusion pressure was varied in

Figure 15

Effect of Perfusion Pressure on Coronary Flow Rate



The lines are drawn by eye, neglecting one point in experiment C.

the course of an experiment, the coronary flow rate at one pressure was reproducible at different times. The constancy of the coronary flow rate, after the initial period of decline, was observed in all six experiments in which accurate measurements were possible. These results are in reasonable accord with those of Fisher and Williamson (1961) who found that a decrease of 37.5% in the coronary flow rate during the first 20 minutes of perfusion was followed by a prolonged period of stability. Zachariah (1960) and Gilbert (1963) observed a decrease of approximately 30% in the coronary flow rate which was measured at the beginning and the end of perfusions lasting 45 minutes and 90 minutes in their respective investigations.

On three occasions, the relationship between the coronary flow rate and the perfusion pressure was studied and found to be linear (Fig. 15). The slopes of the regression lines A,B and C are 0.66 ± 0.01, 0.59 ± 0.01 and 0.68 ± 0.04 ml./g. dry weight/$\frac{min.}{mm.}$/cm. H_2O. Opie (1965) found the slope of the relationship between coronary flow and perfusion pressure to be 0.14 ml./g. wet weight/min./mm. Hg. This value is equivalent to 0.62 ml./g. dry weight/min./cm. H_2O, when the ratio of wet to dry weight is taken to be 6:1.

EVIDENCE OF AEROBIC METABOLISM

It is improbable that the flow of blood through the coronary circulation limits the supply of oxygen to the heart of an animal, even when the animal performs severe exercise. The properties of an isolated perfused heart, which is doing no work in pumping perfusate, are more immediately relevant to the 'in vivo' situation when they are studied in a preparation which is not

hypoxic. For the investigation of the permeation of glucose into cardiac muscle, the state of oxygenation of the heart is of particular significance because anoxia stimulates the permeation of D-xylose into the cells of the isolated diaphragm (Randle and Smith, 1958).

In this work, the adequacy of the supply of oxygen has been assessed from the rate of production of lactic acid by the heart. This method has a particular advantage. When the condition of the preparation remains stable throughout an experiment, the concentration of lactate in the perfusate may be expected to reach a constant level. The rate of formation of lactate is given by the product of this stable concentration and the rate of introduction of the infusate, which does not contain lactate. Because a change in the rate of formation of lactate will be reflected in a proportional change in the concentration of lactate in the perfusate, rather than in the difference of two concentrations as would be the case for a change in glucose utilisation, the concentration of lactate can be, in some circumstances, a better indicator of the stability of the preparation than the concentration of glucose in the perfusate. A high rate of lactate formation was always found when a heart was abnormal in its rate and force of contraction or showed signs of incomplete perfusion as judged by areas of discolouration on the surface of the heart which are palpably harder than their surroundings. However, in some experiments, the concentration of lactate in the perfusate, which was measured at 10 or 20 minute intervals, rose when no visible evidence of a deterioration in the condition of the heart had been observed. The most probable explanation of this observation is that localised anoxia had developed within the heart from the obstruction of part of the coronary

circulation without the occlusion being sufficiently severe to affect the appearance of the preparation. A constant low rate of lactate formation was, therefore, taken as evidence of aerobic metabolism which was maintained throughout an experiment.

Method of Lactic Acid Estimation

L (+) lactic acid has been estimated by the method which was described by Lundholm, Mohme-Lundholm and Vamos (1963). Some minor modifications were made for the sake of experimental convenience. The method depends on the measurement of the extinction at 340 nm. of the reduced nicotinamide adenine dinucleotide (NADH) which is produced in equimolar amounts when lactate is converted to pyruvate in a solution at pH 9.0 which contains L(+) lactic acid dehydrogenase (LDH) from rabbit muscle and an excess of nicotinamide adenine dinucleotide (NAD). The pyruvate is removed from the products of this reaction by its conversion to the hydrazone in the presence of hydrazine.

Reagents:

(i) 0.63 M Glycine Buffer containing 0.4M Hydrazine. The pH of the solution was adjusted to 9.0 with sodium hydroxide.

(ii) 0.03 M NAD (Boehringer) in solution in the glycine buffer.

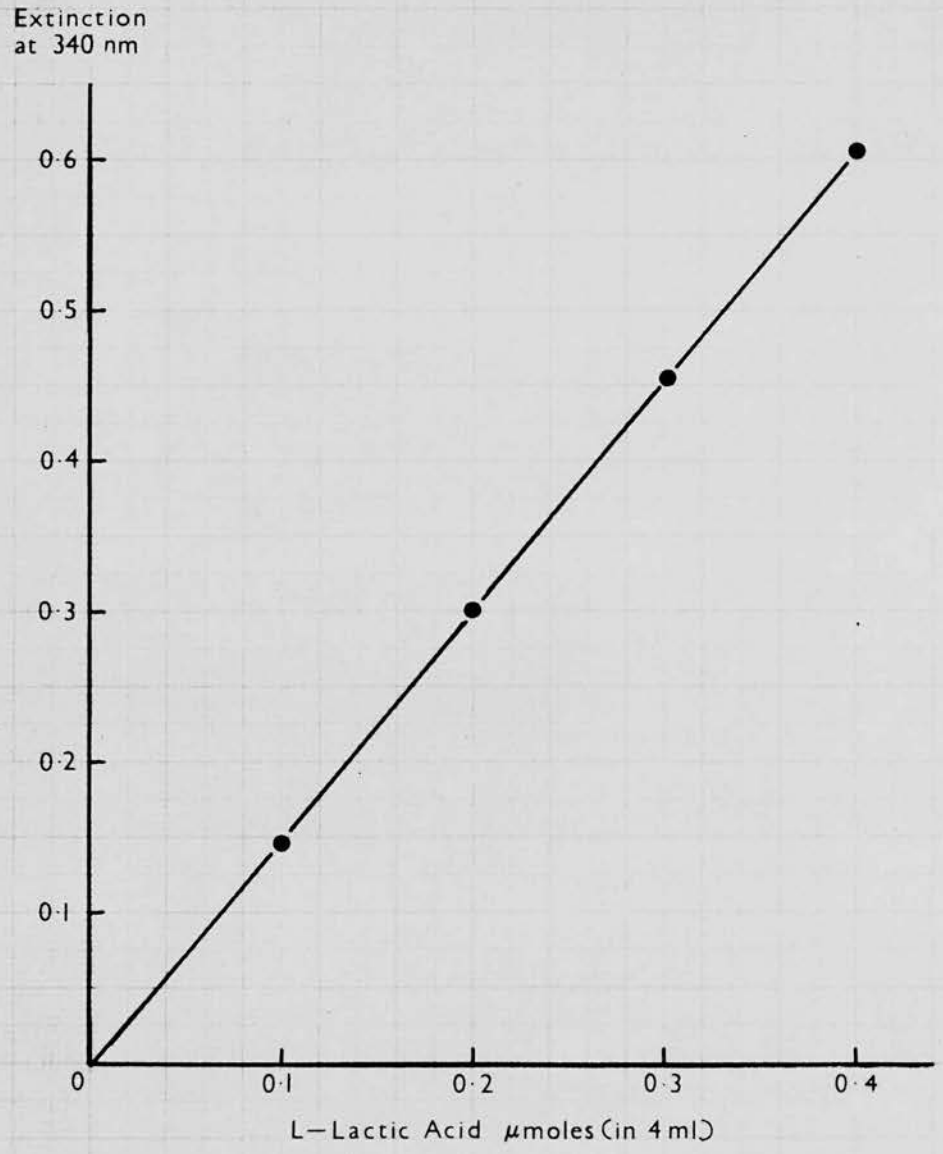
(iii) LDH from rabbit muscle (Boehringer) containing 1 mg. of enzyme protein/ml. The solution was prepared by dilution of a commercial preparation (10 mg. enzyme protein/ml.) with the glycine buffer.

The estimations were conducted and the extinctions at 340 nm were determined in colorimeter tubes which were supplied by Bausch and Lomb for use in the Spectronic 20 colorimeter. The tubes are optically matched within an

Figure 16

ESTIMATION OF L—LACTIC ACID —

Compliance with Beer's Law



The points are the average extinction of duplicated estimations. Extinctions were measured in the Spectronic 20.

average variation of 1%, and have an internal diameter of 1.17 cm. Although the variation in the optical properties of the tubes contributes to the overall error of the estimation, this procedure was preferred because pouring the reaction mixture from one tube to another or shaking the mixture was found to cause a variable increase in the extinction at 340 nm. The increase in extinction could not be attributed to an incomplete mixing of the reagents and its cause remains unknown.

The total volume of liquid in each tube was 4.0 ml. For the determination of the lactic acid in a sample of 0.1 or 0.2 ml., 3.6 or 3.5 ml. of the glycine buffer, 0.2 ml. of the solution of NAD and 0.1 ml. of the solution of LDH were added to a colorimeter tube. A reference tube received glycine buffer in place of the sample of lactic acid. The tubes were incubated at 25°C for 60 minutes. No increase in the extinction, relative to the reference tube, occurred on longer incubation.

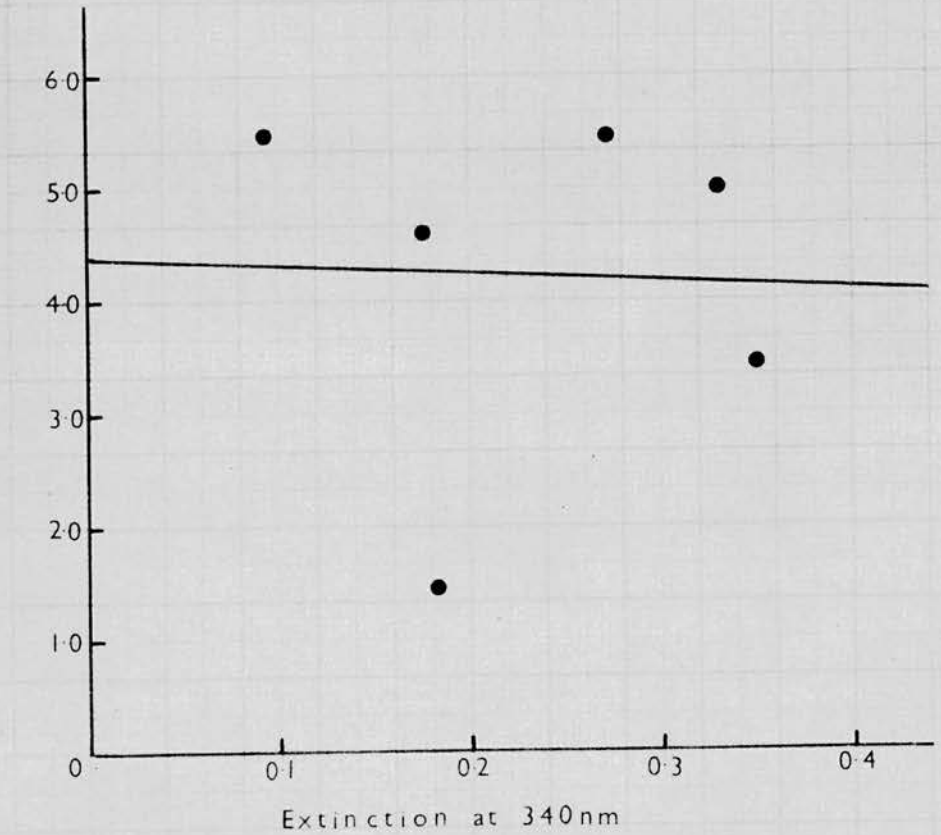
The molar extinction coefficient of NADH was assumed to be 6.22×10^6 cm.^{2} per mole (Kornberg, 1957). On this basis, an increase in the extinction of 0.155 in a tube, which had a light-path of 1 cm. and contained 4.0 ml. of solution, was equivalent to 0.1 μmoles of lactate per tube. Although in the same circumstances, a light-path of 1.17 cm. would give an extinction of 0.181, similar calibration lines were obtained with the Spectronic 20 as with the SP 500 and 1 cm. cuvettes. Bausch and Lomb tubes were therefore used as though their effective light-path was 1 cm. A systematic error arising from this assumption would result in the over-estimation of the concentration of lactate.

Fig. 16 shows the agreement with Beer's law over the range of concentration which was studied. The slope of the regression line in Fig. 16 is

Figure 17

ESTIMATION OF L-LACTIC ACID -

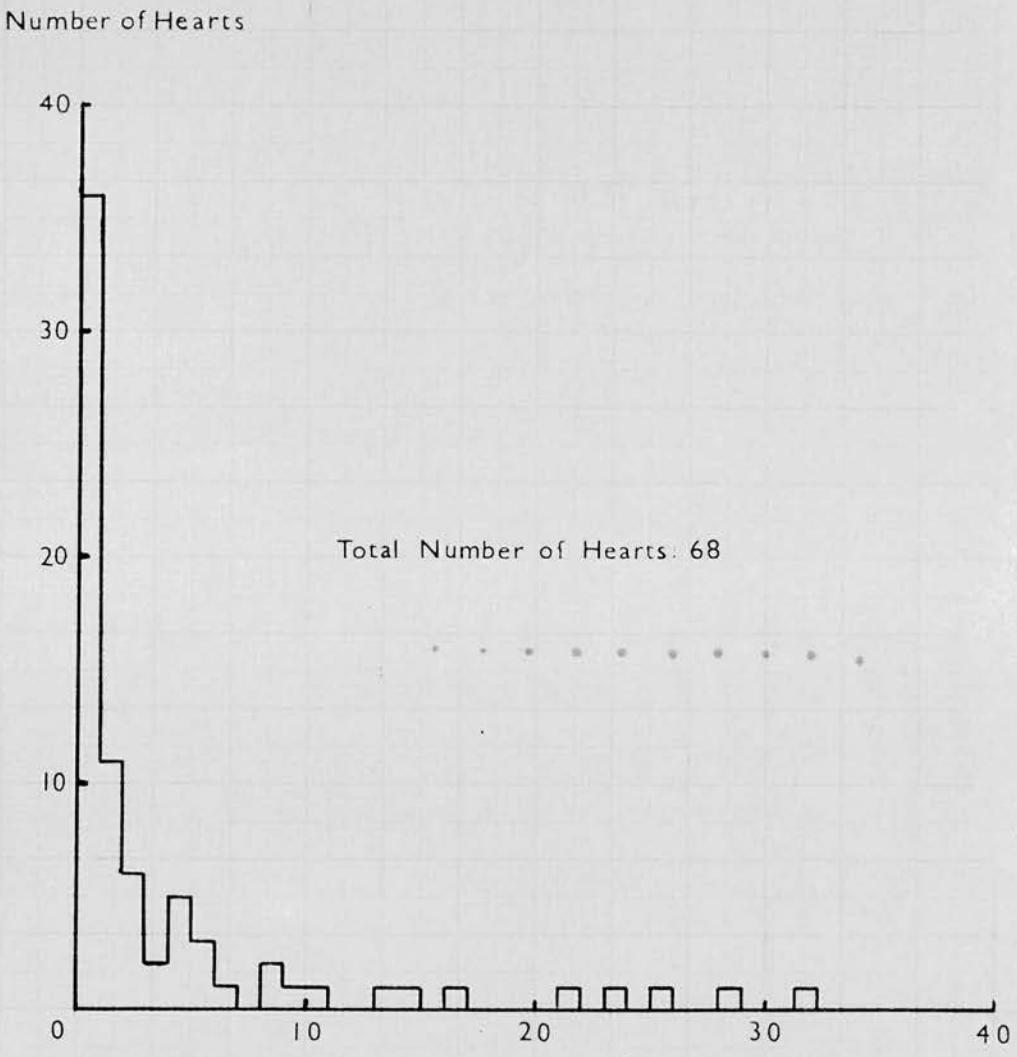
Relation between Extinction and the Standard Deviation of the Extinction



Each point shows the mean and standard deviation of six estimates.
The slope of the regression line does not differ significantly from zero.

Figure 18

Distribution of Lactate Formation among Hearts Perfused Without Insulin



1.53 ± 0.02 and the intercept does not differ significantly from zero. It can be concluded from Fig. 17 that the standard deviation of any one extinction is independent of its magnitude and has an average value of 0.004 µmoles (in 4.0 ml. of reaction mixture). This method of lactate estimation is therefore only precise when the amount of lactate in the reaction mixture is greater than 0.1 µmole and therefore (if 0.2 ml. of perfusate is taken for analysis) when the concentration of lactate in the perfusate is greater than 0.5 µmole /ml. (4.5 mg./100ml.). Since the infusion rate was 0.5 ml./min. in the majority of this work, the minimum rate of lactate formation which could be measured accurately was 1.35 mg./hour, which is equivalent to 8.5 to 10 mg./g. dry weight/hour for the hearts in this work.

Results

In the majority of experiments, the concentration of the perfusate lactate was so low that only qualitative estimates of the rate of formation could be made. When the concentration of lactate in the perfusate was less than approximately 0.5 mg./100 ml., the method for the estimation of lactate did not permit the confident discrimination of the extinctions of the reference and unknown solutions. In the conditions of the experiments, the formation of approximately 1 mg. of lactate/g. dry weight/hour was the minimum rate which could be measured with an error of less than 30%.

Fig. 18 is a histogram of the distribution of observed rates of lactate formation amongst 68 hearts which were perfused without insulin and at concentrations of perfusate glucose between 3 and 300 mg./100ml. It will be shown in section IV that the rate of glucose utilisation in the absence of

insulin is insensitive to the concentration of perfusate glucose, in comparison with the sensitivity in the presence of the hormone. A dependence of the distribution of the rate of lactate formation on the rate of glucose utilisation in the experiments depicted in Fig. 18 is improbable. In 62% of the experiments, lactate formation was less than 1 mg./g. dry weight/hour and in the conditions of the analysis, effectively undetectable.

No reports of rates of lactate formation which are lower than those that have been observed in comparable circumstances in this work have been found in the literature. It was therefore concluded that the hearts which have been perfused in the novel apparatus were adequately supplied with oxygen.

CHAPTER SIX

S U M M A R Y

1. The investigation of the permeation of glucose in the isolated perfused rat heart, which is maintained in a steady state by the infusion of a solution of glucose into a constant volume of recirculated perfusate, requires that the volume of perfusate which is recirculated in a suitable apparatus is no more than 8 to 9 ml.

2. Established systems of cardiac perfusion were considered to be unsuitable for their adaptation to meet the requirement of this investigation, either because the volume of perfusate which the system required was too large or because the system did not regulate satisfactorily the conditions of the perfusion.

3. A novel apparatus has been described which permits a heart to be perfused with 6 ml. of recirculated perfusate while the temperature and the pressure of the perfusion and the oxygenation and filtration of the perfusate are controlled.

4. The rates of contraction, of the flow through the coronary circulation, and the formation of lactate in the rat's heart which is perfused in the novel apparatus are comparable with the properties of hearts which have been perfused in other systems.

S E C T I O N II

CHARACTERISTICS OF THE APPROACH TO A STEADY STATE OF GLUCOSE UTILISATION IN THE PERFUSED HEART

CHAPTER ONE

INTRODUCTION

A steady state of glucose permeation and utilisation in the perfused heart provides a suitable circumstance for the investigation of the kinetics of the process of permeation. The construction and properties of an apparatus, in which the utilisation of glucose might be measured in a steady state, were described in Section I. In this Section, evidence will be presented from which it may be concluded that hearts which are perfused in this apparatus attain a steady state of glucose utilisation and that the rate of utilisation may be accurately measured.

The second chapter of this Section is devoted to the description of the methods by which the concentration of glucose in the perfusate has been estimated. Precision in the analysis of glucose is of particular importance for this investigation. The determination of glucose utilisation by a perfused heart in a steady state which is maintained by the infusion of glucose into a constant volume of recirculated perfusate depends on the accurate estimation of the difference between the concentrations of glucose in the infusate and the perfusate. This difference will be small at a high rate of infusion, so that the accuracy with which small differences in glucose concentration can be measured limits the practicable rates of infusion. Because the half-time of the approach to a steady state depends on the ratio

of the volume of recirculated perfusate to the rate of infusion, the precision of the estimation of glucose also affects the period of perfusion within which a steady state can be usefully established. With the interaction of these factors, it is equally true to say that, provided the half-time of the approach to a steady state remains acceptable, the need for a small volume of recirculated perfusate is reduced when small differences in glucose concentration can be measured precisely. Of possibly greater significance is the limitation, which is imposed by the accuracy of the estimation of differences in glucose concentration, on the range of concentrations of perfusate glucose which can be studied. The relationship between glucose utilisation and the concentration of perfusate glucose is not linear (Morgan, Henderson et al., 1961) and the rate of utilisation approaches a maximum with increasing concentration, so that the fractional difference between the concentrations of infusate glucose and perfusate glucose will fall as the concentration of perfusate glucose is increased unless the infusion rate is also varied. These considerations were predominant in the choice and development of methods for the estimation of glucose, although it became apparent in the course of the investigation that the study of glucose utilisation at low concentrations of perfusate glucose might be of particular value in the determination of the parameters of glucose permeation in rat cardiac muscle (Section IV).

In the third chapter of this Section, the results, which were obtained when the concentration of perfusate glucose was determined either continuously or periodically during the course of an experiment, are presented. When the uptake of glucose by a perfused heart may be expected to be unaffected by a small change in the concentration of the perfusate glucose, the time-course

of the approach to a constant concentration should be described by a single exponential. The observation by Zachariah (1961) and Gilbert (1963) of changes in the permeability of the isolated perfused heart to non-metabolised sugars during the first 30 minutes of a perfusion without insulin suggested that the approach to a steady state in the perfusate glucose concentration, when a heart is perfused without insulin, might be more complex than that predicted by a single exponential change. This complexity was found.

The fourth chapter continues the analysis of the implications of the changes in the concentration of perfusate glucose during the course of a perfusion. A particular advantage of the system for cardiac perfusion which was developed for this work is that it is possible to determine the rate of change of glucose concentration in the perfusate at any instant in time. Since the rate of utilisation of glucose at any instant is a function of the glucose concentration and of the rate of change of the glucose concentration at that time, the rate of glucose utilisation can be followed from instant to instant throughout the experiment. The theoretical basis for this procedure is set out in Chapter Four. The utilisation of glucose by hearts which were perfused in the absence of insulin was found to vary markedly during the period of perfusion.

The significance of the results which will be presented in this Section and the extent to which the conditions for the investigation of the kinetics of glucose permeation have been satisfied are discussed in chapter five.

CHAPTER TWO

THE ESTIMATION OF GLUCOSE

INTRODUCTION

The necessity for a precise method for the estimation of glucose has been set out. There is also potential advantage in the rapid estimation of glucose. If the concentration of perfusate glucose could be determined quickly and, preferably, by a system in which the concentration is measured and recorded continuously, the establishment of a constant concentration of glucose in the perfusate of a heart could be recognised while perfusion continues. Such a facility would reduce the unnecessary protraction of an experiment whose object is satisfied when a steady state of glucose utilisation exists. Alternatively, the effects on a steady state of the introduction to the system of, for example, a hormone, a second nutrient, or a drug could be more usefully investigated when a departure of the system from a steady state could be recorded. An attempt to develop a system for the continuous and rapid estimation of glucose is described in the following chapter.

With this application in mind, the method for the estimation of glucose, which was used in the early stages of this investigation, was chosen and developed for both precision and rapidity. The method depends upon the catalysis by β-D-glucose oxidase (EC 1.1.3.4) of the oxidation of β-D-glucose

by oxygen with the formation of D-glucono-5-lactone and hydrogen peroxide and the subsequent oxidation of a chromogen, o-tolidine, by the hydrogen peroxide, in the presence of peroxidase (EC 1.11.1.7). When the carcinogenic property of o-tolidine was publicised, a more wholesome chromogen was sought. Gum guaiacum resin proved to be a suitable substitute. Both of these methods for glucose estimation have been adapted for the requirements of this investigation from procedures which have been published by other workers.

AUTOMATED ANALYSIS

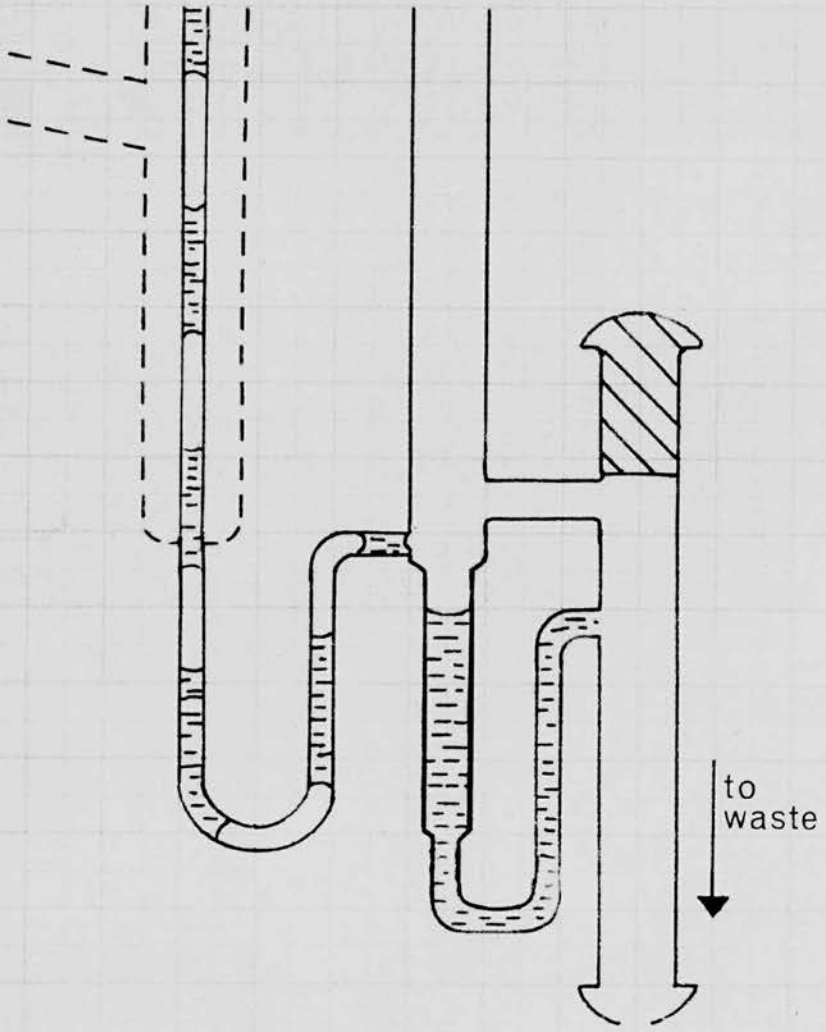
All estimations of glucose, other than those made by a system for the continuous monitoring of perfusate glucose, have been made with the Auto-analyser (Technicon Instruments Co. Ltd. Chertsey, Surrey). Automated analysis allows a large number of estimations to be made with a precision which is not affected by variations in the proficiency of the operator. This is especially advantageous when the product of a reaction is unstable, as is the case for the products of the oxidation of o-tolidine and gum guaiacum resin. With a manual method, such instability necessitates the careful timing of the reading of the extinction of each reaction mixture. With an automated method, the identity of the reaction times of all estimations is achieved as a necessary property of the system. For the comprehension of the modifications which have been made in a manual method, a brief description of the principle of the Auto-analyser will be given.

The Auto-analyser is a continuous flow system for which the motive force is provided by a peristaltic proportioning pump. Rollers compress and travel

along up to eight plastic tubes lying parallel to one another. As the flows from the tubing can be combined, the availability of Tygon tubing on seventeen different cross-sections permits a virtually unrestricted range of total flow rate. In the simplest arrangement of the Autoanalyser, three tubes are required. Through one tube a sample is aspirated from one of a series of cups in a sampler module, which is designed to switch from one cup to the next at regular intervals. Through a second tube, reagent is aspirated from a reservoir and, through the third, air or some other appropriate gas is aspirated. The flows of gas, sample and reagent are combined so that the reaction mixture is regularly segmented by bubbles which, by disturbing the parabolic flow profile of the liquid between the axis and the periphery of the tubing throughout the apparatus, reduce the interaction between the segments and therefore between successive samples.

A homogeneous reaction mixture is established when the segmented stream passes through a coil whose longitudinal axis is horizontal. A segment is mixed as the stream is repeatedly inverted with the turns of the coil. The mixing-coils which were used in this work have a capacity of 4.0 ml. Passage of the reaction mixture through other coils in a thermostatically regulated bath for a suitable period precedes the measurement of some property of the mixture. It is not necessary for a reaction to reach equilibrium before a measurement is made because of the identity of the reaction times of all estimations. In this work, the coloured products of a reaction were measured in a flow-cell of a colorimeter in which the liquid is automatically separated from the gas. A recorder registers the per cent transmittance of light through the cuvette of the flow-cell.

Figure 19.



The Modified Flow-Cell

The part of the original cell which is omitted is represented by the interrupted lines.

A modification has been made to the flow-cell which is supplied by the manufacturer of the Autoanalyser. In the original flow-cell, the gas bubbles separate from the reaction mixture in a chamber before the cuvette. The omission of this chamber (Fig. 19) and the retention of the segmentation of the stream until the cuvette is reached, reduce considerably the volume with which the incoming liquid must exchange. The attainment of a solution of constant transmittance in the cuvette is hastened by the modification, which allows bubbles to separate from the reaction mixture without interrupting the light path.

The extent of the exchange of the contents of the flow-cell in each cycle of operation depends upon the volume of reaction mixture of constant composition which can be introduced into the cell and the volume of liquid held by the cell. Only the former factor can be varied either through the alteration of the flow rate or through the alteration of the period during which a sample is aspirated. The practicable rate of flow is restricted by the availability of sample, the cost of a reagent and the concentration of a coloured product. The sampler module limits the longest period of aspiration of a sample to 2 minutes. These restrictions make it impossible to prevent the interaction of successive samples in the flow-cell. However, when alternate cups in the sampler module contain distilled water, there is no interaction between the remaining samples at any of the three possible rates of sampling. At the slowest rate only 10 estimations in the hour can be made when alternate cups contain water. At the fastest rate, each sample is aspirated for only 40 seconds so that the per cent transmittance of the contents of the cuvette is always changing rapidly and reproducible traces cannot be obtained on the

recorder. The intermediate rate of 40 aspirations to the hour allows 20 estimations to be made and, since 2 to 1 is the ratio of the time during which sample is aspirated to that when air is aspirated in the switch from cup to cup, this allows exchange of the contents of the flow-cell to occur for 1 minute. Automated methods for glucose estimation have been developed empirically to achieve, at a rate of 20 estimations per hour, an exchange of the contents of the flow-cell which is sufficient to give reproducible traces on the recorder without loss in the sensitivity of the methods through the dilution of the reaction products in too large a volume of reaction mixture.

Treatment of Data from the Autoanalyser

Error can be introduced in the interpretation of the values for the per cent transmittance, which are obtained with the Autoanalyzer, when the base-line trace on the recorder varies in the course of a series of estimations. The variations in the base-line extinction are very nearly linearly dependent on time and are usually caused by progressive changes in the properties of a reagent or by deposition in the flow cell. These effects have been countered by making each estimate in duplicate in a series of standard solutions (S1-3) and solutions of unknown concentration (U1-4) so that the whole has mirror symmetry:

x S1 x S2 x S3 x U1 x U2 x U3 x U4 x U4 x U3 x U2 x U1 x S3 x S2 x S1 x

where x represents a sample of distilled water. The arithmetic means of the pairs of extinctions all refer to the mid-point in time in the series, whereby the influence of any regular change in the base-line is nullified.

In practice, the per cent transmittances were read from the recorder and

converted into units of extinction. The extinctions of duplicate estimations were summed and the coefficients of a linear regression of concentration on extinction were calculated from the extinctions and concentrations of the standard solutions. The concentrations which corresponded with the extinctions given by the solutions of unknown concentration were then determined from the coefficients of the regression. This procedure, from the conversion of per cent transmittance into extinction to the final calculation of the concentration of the unknown solution, was carried out in the later stages of this investigation on an Olivetti Programma 101 with a programme written by Mr. I.A. Nimmo, to whom I am grateful.

The computation of the regression of concentration on extinction rather than of extinction on concentration is justifiable because the variance in these regressions is always small. Thus in three instances, which were taken at random, the products of the slopes of the two regressions were 0.99984, 0.99977 and 0.99996. From this it follows that either regression coefficient can be estimated to within one part in 10^3 by taking the reciprocal of the other.

ESTIMATION OF GLUCOSE WITH o-TOLIDINE AS CHROMOGEN

The method which has been most used for the enzymic estimation of glucose is that of Huggett and Nixon (1957). In this method, the hydrogen peroxide which is formed when glucose oxidase catalyses the oxidation of glucose oxidises o-dianisidine in the presence of peroxidase. The product of this reaction, which is conducted in a phosphate buffer, pH 7.0, at $37^{\circ}C$ is stable

when its tendency to precipitate is reduced by the inclusion in the reaction mixture of the iso-octyl phenyl ether of polyethyleneglycol - Triton X-100 (Dahlqvist, 1964). Consequently, this procedure is well suited to unautomated use. For the rapid estimation of glucose in a system for the continuous monitoring of the concentration of glucose in the perfusate of a perfused heart, the method of Huggett and Nixon is unsuitable because the development of colour is slow and is complete only after incubation for an hour.

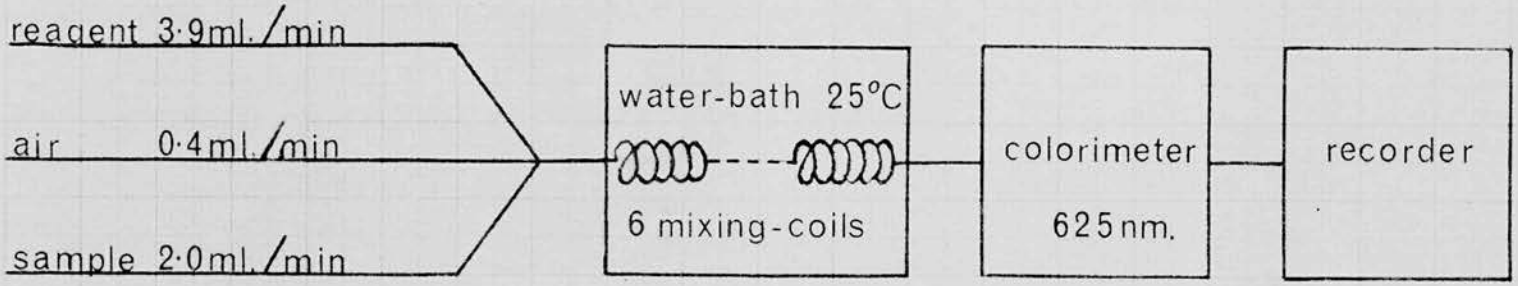
Middleton and Griffiths (1957) developed a reagent for the estimation of glucose which was similar to that of Huggett and Nixon except for the substitution of o-tolidine for o-dianisidine. This modification gave the advantage of greater rapidity in the development of colour but the instability of the coloured product limited the unautomated use of the method. However, the reagent itself is too unstable for the method to be used with the Autoanalyser for periods of more than an hour.

A stable reagent with o-tolidine as the chromogen was introduced for the estimation of glucose by Marks (1959), who replaced the phosphate buffer, pH 7.0, by a 0.15 M acetate buffer, pH 5.0. At room temperature the development of colour with this reagent reached a maximum after 12 minutes and thereafter with the Autoanalyser.

Stock Solutions for the Reagent

1. Sodium Acetate Buffer, 0.15 M, pH 5.0.
2. A 1% (w/v) solution of o-Tolidine in absolute ethanol, which is stable for at least 3 months at 4°C.
3. A 20% (v/v) solution in absolute ethanol of Triton X-100 (supplied by

Figure 20



System for the Automated Estimation of Glucose

Lennig Chemicals Ltd.), which is stable indefinitely at 4°C.

4. A 0.1% (w/v) solution of horse-radish peroxidase in distilled water, which is stable for 2 weeks at 4°C.
5. Fermcozyme: a commercial preparation which contains 0.75 mg. of glucose oxidase per ml. It is stable for at least 6 months at 4°C.

Peroxidase and Fermcozyme were both obtained from Hughes and Hughes Ltd.

Composition of the Reagent

To prepare 1 litre of reagent, 10.0 ml. of Fermcozyme, 10.0 ml. of the stock solution of peroxidase, 10.0 ml. of the stock solution of o-tolidine and 1.0 ml. of the stock solution of Triton X-100 were added to approximately 500 ml. of the acetate buffer. The reagent is made up to volume with the buffer and is stable for at least a week when it is stored at 4°C.

The System for Automated Estimation of Glucose

Fig. 20 depicts the system whereby glucose was estimated with the Auto-analyser, with o-tolidine as the chromogen. 2.0 ml./min. of the solution of glucose and 3.9 ml./min. of the reagent were combined and the stream was segmented with air (0.4 ml./min.). Passage through a series of six mixing-coils and their connections established the reaction mixture and allowed a reaction time of 4.5 minutes. The coils were immersed in a water bath in which a Circotherm maintained a temperature of 25°C. The reaction mixture was delivered to a flow-cell with a 1 cm. light-path and the per cent trans-mittance of light at 625 nm was recorded.

The Characteristics of the Method

1. The Effect of Temperature and Reaction Time

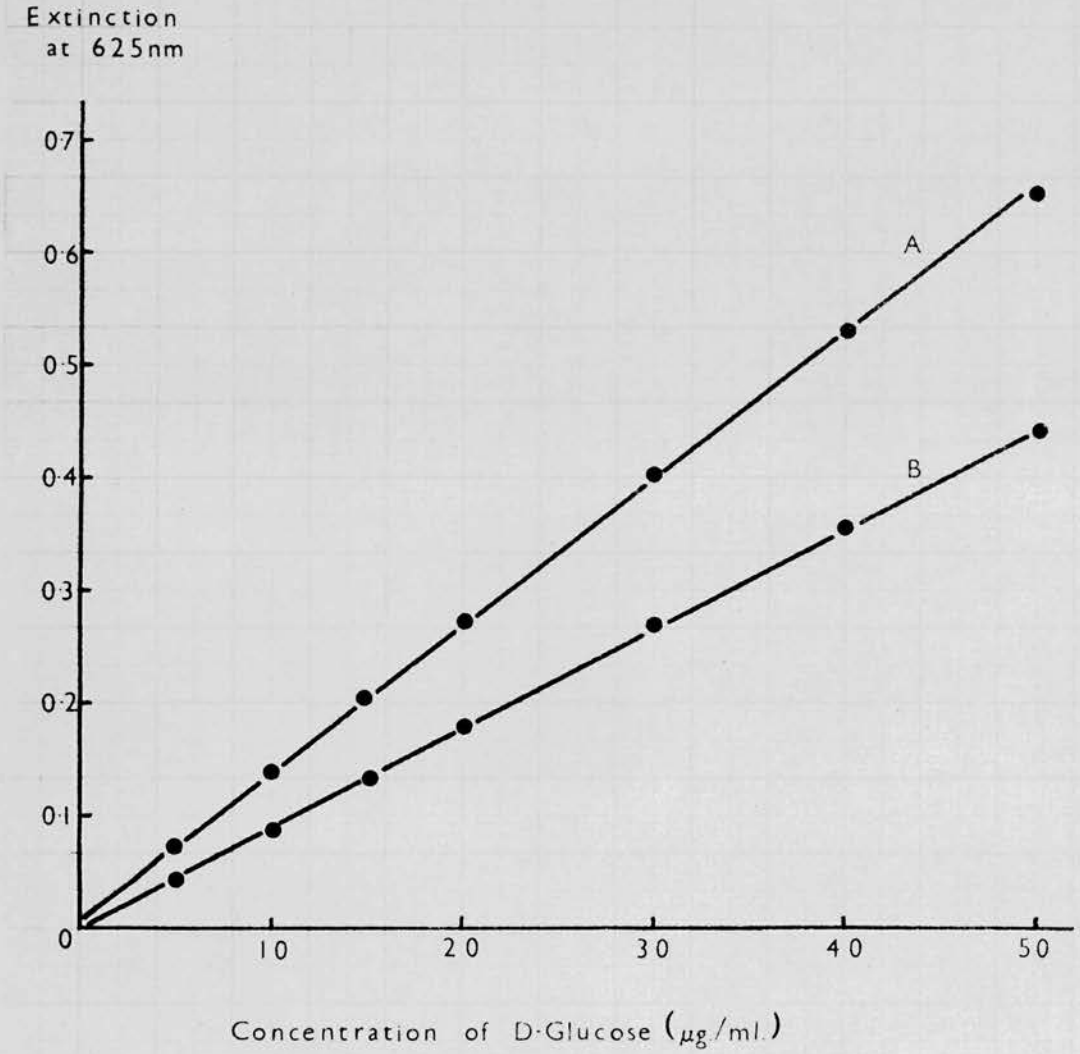
No attempt was made to obtain any more than a qualitative understanding of the influence of temperature and the period of reaction on the intensity of the colour which was developed with this method. A reaction time of 4.5 minutes at 25°C produced the greatest intensity of colour. Addition to or subtraction from the six coils, through which the reaction mixture was passed, reduced the colour which was developed at a particular concentration of glucose. The temperature, at which the reaction was conducted, had little effect, within the range 20 to 40°C, on the intensity of colour, but the higher temperatures caused slight decreases in intensity. A temperature of 25°C was chosen for the routine use of the method, because it is sufficiently greater than room temperature to be easily regulated.

The discrepancy between the times (4.5 minutes and 12 minutes) for the maximum development of colour with this method and the unautomated method of Marks (1959) may be attributed to a difference in the amounts of glucose oxidase which were used. The reagent which has been described contains twice the volume of Fermcozyme which was included in the reagent of Marks. It was sometimes convenient in this work to employ a reagent in which the amounts of Fermcozyme, peroxidase and o-tolidine were halved. The extinctions observed with this dilute reagent, in the system of Fig. 20, were less than those produced by the normal reagent and enabled higher concentrations of glucose in the sample solution to be estimated accurately, as will be shown later. The discrepancy might also be thought to reflect the better oxygenation of a reaction mixture when it is segmented by air, because the

Figure 21

ESTIMATION OF D—GLUCOSE

Compliance with Beer's Law when o-Tolidine is the Chromogen



Plot A was obtained with a reagent which contained twice the concentrations of glucose oxidase and peroxidase which were present in the reagent that yielded plot B.

consumption of oxygen in the reaction might affect the Km of glucose oxidase for glucose, which depends upon the partial pressure of oxygen (Laser, 1952). However under no circumstances in which the method of Marks might be used would the partial pressure of oxygen be reduced by more than 1% which would not significantly affect the Km of glucose oxidase.

2. The Effect of pH

The stability of the product of the oxidation of o-tolidine by hydrogen peroxide in the presence of peroxidase increases as the pH of the solution is reduced from 5.0 to 3.0 (G.L. Atkins - personal communication). When the reagent for the estimation of glucose was prepared in a 0.15 M acetate buffer, pH 4.0, the intensity of the colour development in the system illustrated in Fig. 20 was reduced. The stability of the colour and the effect of a longer reaction time at pH 4.0 were not investigated because the stability of the reaction product is irrelevant to the development of an automated method of analysis and, in this case, rapidity in the generation of colour was a major consideration. However, the reduction in the intensity of colour which was developed at pH 4 presumably reflects the decreased activity of glucose oxidase (optimum pH 5.6).

3. Compliance with Beer's Law

The relationship between the extinction at 625 nm and the concentration of glucose in the sample is shown in Fig. 21 for the reagent which was described on p. 73, and for the reagent in which the content of glucose oxidase, peroxidase and o-tolidine were halved. Within the range of concentration of glucose which was studied, the compliance with Beer's Law is good.

The omission of Triton X-100 from the reagent has no effect on the

relation between the extinction and the concentration of glucose. Triton X-100 was included in the reagent because it had a beneficial effect in an apparatus which was designed to measure continuously the concentration of perfusate glucose and which will be described in the following chapter. The same reagent was used in both systems for administrative convenience.

4. The Effect of Procedures for Deproteinisation

a) Metaphosphoric acid

The inclusion of 2 ml. of 20% metaphosphoric acid in a solution of glucose, which had a final volume of 25 ml., resulted in a distortion of the traces on the recorder of the Autoanalyser. Estimates of the per cent transmittance could not be made.

b) Cadmium sulphate and sodium hydroxide

The precipitation of cadmium hydroxide in a solution of glucose by the titration, to a phenolphthalein end-point, of 2 ml. of 6.5% cadmium sulphate with approximately 0.25 N sodium hydroxide reduced the apparent concentration of glucose in the filtrate to 73% of the true concentration. Addition of barium carbonate (Miller and van Slyke, 1936) after the precipitation of cadmium hydroxide did not result in any improvement.

c) Zinc sulphate and sodium hydroxide

The precipitation of zinc hydroxide in a solution of glucose did not result in a discrepancy between the apparent and the true concentration of glucose in the filtrate. In the standard procedure, which was adopted for this work, 2 ml. of 5% zinc sulphate were added to a sample of the perfusate, which had been diluted with about 20 ml. of water, or whatever volume was practicable for a smaller overall dilution. The solution was titrated slowly

TABLE 3

EFFECT OF DEPROTEINISATION WITH ZINC HYDROXIDE ON THE ESTIMATION OF GLUCOSE WITH o-TOLIDINE AS CHROMOGEN

Zinc hydroxide was precipitated (from 2 ml. 5% $ZnSO_4$) in six solutions of glucose of known concentration. The apparent concentration of glucose in the filtrates of these solutions was determined.

Added glucose (µg./ml.)	5.0	10.0	15.0
Estimated glucose (µg./ml.)	5.08 5.01	9.99 9.96	15.04 14.96
Mean estimate	5.05	9.98	15.00
Percent. recovery	101.0	99.8	100.0

TABLE 4

THE ACCURACY OF THE ESTIMATION OF GLUCOSE CONCENTRATION WITH o-TOLIDINE AS CHROMOGEN

Five estimates were made of the concentration of glucose in a deproteinised solution. The mean and standard deviation of these estimates are shown for eight instances. The mean standard deviation is 0.07 μg./ml. The percent. error is also given.

Mean estimate (μg./ml.)	Standard deviation (μg./ml.)	Percent. error
10.02	0.06	0.60
10.42	0.08	0.77
14.89	0.13	0.87
14.97	0.05	0.33
14.99	0.06	0.40
15.85	0.09	0.57
19.79	0.06	0.30
20.05	0.06	0.30

TABLE 5

EFFECT OF INULIN ON THE ESTIMATION OF GLUCOSE WITH o-TOLIDINE AS CHROMOGEN

The apparent concentration of glucose was measured in nine solutions containing known amounts of glucose and inulin.

Added glucose (μg./ml.)	Estimated glucose (μg./ml.) in solutions containing:		
	100 μg. inulin/ml.	200 μg. inulin/ml.	300 μg. inulin/ml.
5.0	5.02	5.00	4.96
10.0	10.02	10.03	10.00
15.0	14.92	14.89	14.83

with approximately 0.25 N sodium hydroxide in the presence of phenolphthalein, diluted to a volume which was known and filtered. Table 3 shows that the apparent concentrations of glucose in six filtrates, which were prepared in this way, agree with the true concentrations of glucose in these solutions.

5. The Accuracy of the Estimates of Glucose Concentrations

In Table 4 the mean value and the standard deviation from the mean of repeated estimations of the concentration of glucose in eight solutions are presented. The estimations were made on filtrates which were obtained when samples of infusate or recirculated perfusate were deproteinised by the precipitation of zinc hydroxide. It can be concluded from the table that the standard deviation is independent of the magnitude of the concentration between 10 and 20 µg./ml. and has an average value of 0.07 µg./ml. This range of concentrations covers the great majority of the solutions which have been estimated in this work.

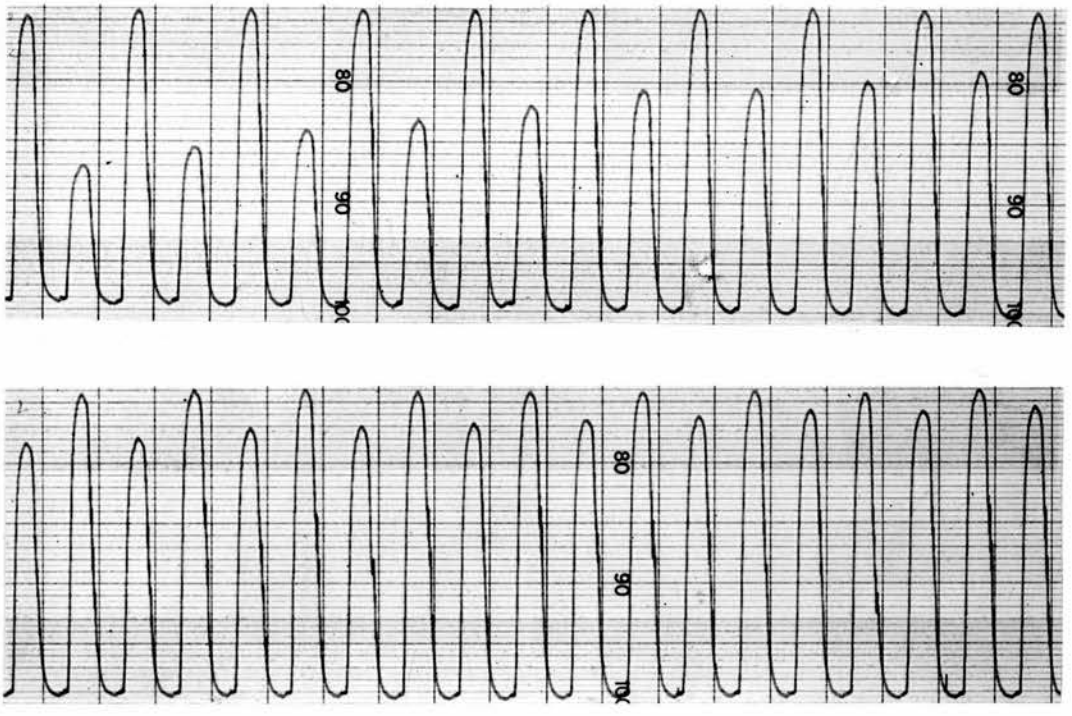
6. The Effect of Inulin on Glucose Estimations

Although both inulin and raffinose were used for the determination of extracellular water, only inulin was employed in those experiments for which the estimation of glucose was made with the reagent in which o-tolidine was the chromogen. The effect of inulin on the estimation of glucose was investigated by the determination of the apparent concentration of standard solutions of glucose in which inulin was included. The highest concentration of inulin in the solutions of glucose exceeded that which might occur after the dilution of a sample of perfusate for the analysis of glucose. Table 5 shows the results of this experiment. No effect of inulin on the estimation of glucose was detected.

Fig. 22

ESTIMATION OF D-GLUCOSE

The effect of mutarotation when o-tolidine is the chromogen



The upper and lower traces were continuous. Two solutions of the same concentration of glucose were aspirated alternately in the AutoAnalyser. One, at mutarotational equilibrium, gave reproducible peaks. The other, in which α-glucose exceeded equilibrium amounts, gave lower but increasing peaks as equilibrium was approached. The experiment is described in more detail in the text.

7. The Preparation of Standard Solutions of Glucose

The glucose oxidase in the reagent for glucose estimation is specific for β-glucose and the estimation is conducted at 25°C, with a reaction time of 4.5 minutes. Because the solutions of glucose were prepared from α-glucose, it was necessary, under these conditions, to ensure that α- and β-glucose had equilibrated in a solution which was to be analysed. The mutarotation of a solution of α-glucose can be followed readily. In Fig. 22 the peaks of constant transmittance were produced by a freshly prepared solution of α-glucose which was heated in a boiling water bath for 15 minutes before it was cooled to room temperature and analysed. The alternate peaks of decreasing transmittance were produced by a solution of α-glucose whose concentration was the same and which was treated identically except that it was placed in a refrigerator while the other solution was heated. The per cent transmittances obtained with the two solutions could still be distinguished after the solutions had stood for two hours at room temperature. Consequently, the stock solutions of glucose, from which the standard solutions were prepared, were always heated for 15 minutes in a boiling water bath.

8. Summary

The automated enzymic method for the estimation of glucose, in which o-tolidine is the chromogen, is sensitive and precise. A maximum development of colour is reached quickly. Solutions of glucose can be deproteinised by the precipitation of zinc hydroxide without affecting the accuracy with which their concentrations are determined. The inclusion of inulin does not affect the estimation of glucose.

The method is therefore suitable for the accurate measurement of small

differences in glucose concentration and is also suitable for use in a system for the continuous measurement of the concentration of perfusate glucose.

THE ESTIMATION OF GLUCOSE WITH GUM GUAIACUM RESIN AS CHROMOGEN

The carcinogenic property of o-tolidine mars an otherwise excellent method for the estimation of glucose. An alternative chromogen was therefore sought. A reagent for the estimation of glucose with gum guaiacum resin as the chromogen has been described by Hill and Cowart (1966). The system, which was illustrated in Fig. 20 for the estimation of glucose in the Autoanalyser with o-tolidine as the chromogen, proved to be suitable for the reagent of Hill and Cowart with two modifications. The number of mixing coils through which the reaction mixture was passed was increased to seven, thereby raising the reaction time to about 5 minutes, and the temperature of the water bath, in which the coils were immersed, was raised to 37°C.

The Stock Solution of Gum Guaiacum Resin

Gum guaiacum resin was obtained through J.H. Bairds, Ltd. Edinburgh and from Brome and Schimmer, Ltd., London. For 1 litre of the stock solution, 0.75 g. of the finely ground resin were added to 12.5 ml. of a solution of 95% ethanol in water. Approximately 75% of the resin is soluble in ethanol. The ethanolic solution was filtered into 600 ml. of 1M sodium acetate buffer, pH 5.6, which contained 20 ml. of Triton X-100, and the whole was diluted to 1 litre with distilled water.

Although less than 20 ml. of Triton X-100 was sufficient to maintain

the alcoholic extract of the resin in solution in the acetate buffer, the whole volume of Triton X-100 was necessary to prevent the precipitation of the product of the oxidation of the chromogenic component of the resin from the reaction mixture.

The Preparation of the Reagent

For 1 litre of reagent, 670 ml. of the solution of the resin were diluted with 330 ml. of water which contained 10 ml. of Fermcozyme and 10 mg. of horse-radish peroxidase.

The Properties of the Method

1. General

The description of the characteristics of the method is complicated by their partial dependence on the source and the batch of the resin from which the reagent was prepared. With one batch, it was found that the stock solution of the resin gave good results only after it had been left in solution at room temperature for a week to ten days. The graphical relation between extinction and the concentration of glucose in the sample was then linear, at low concentrations of glucose, but had a large negative intercept on the ordinate. When 2.5 µg. of glucose were added to each ml. of the reagent and the resultant colour was allowed to fade, the subsequent use of this reagent gave a relation between extinction and the concentration of glucose which was linear at low concentrations of glucose and had a small positive intercept on the ordinate. The amount of glucose which was added to the reagent had been calculated to be equivalent to that necessary to

Figure 23.

ESTIMATION OF D-GLUCOSE

Compliance with Beer's Law when Gum Guaiacum is the Chromogen

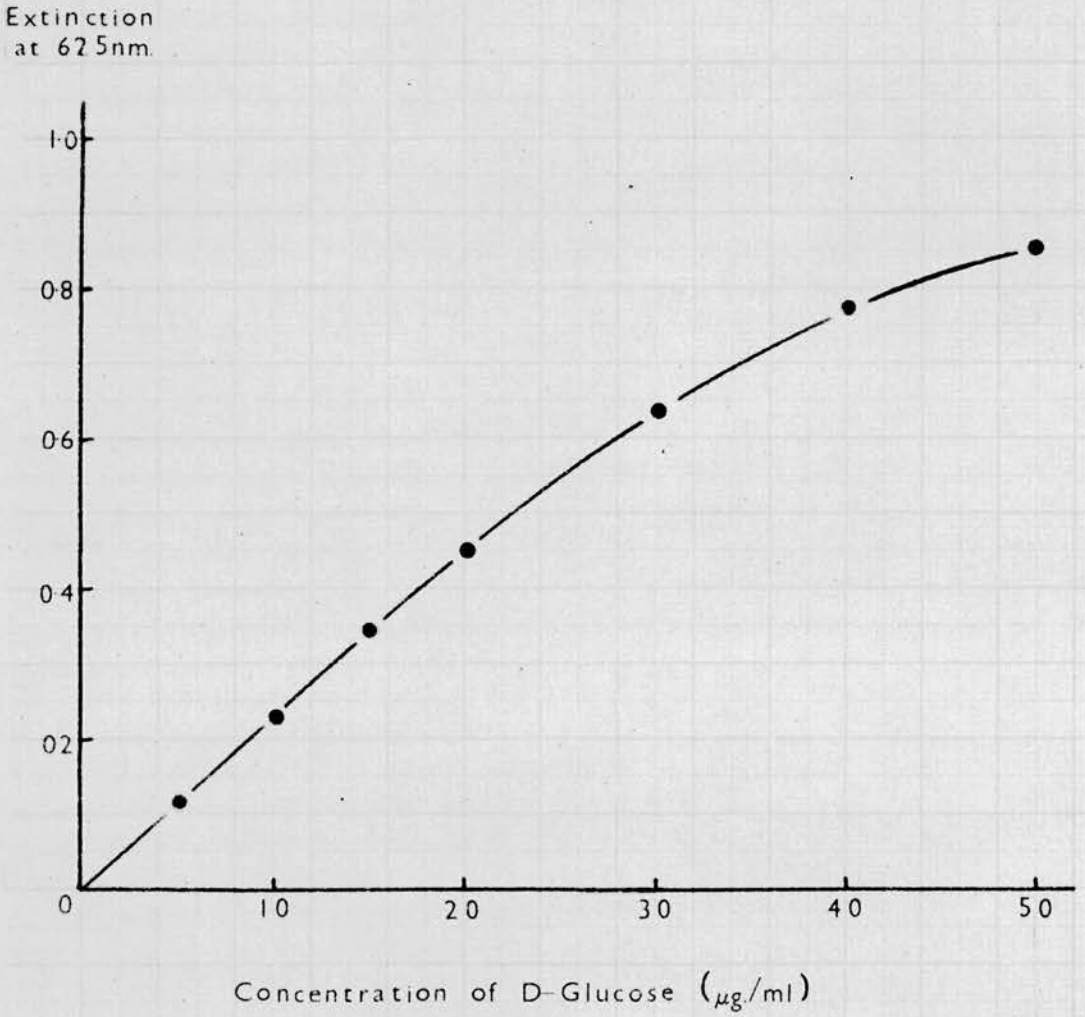


TABLE 6

EFFECT OF DEPROTEINISATION WITH ZINC HYDROXIDE ON THE ESTIMATION OF GLUCOSE WITH GUM GUAIACUM RESIN AS CHROMOGEN

Zinc hydroxide was precipitated (from 2 ml. of 5% $ZnSO_4$) in four solutions of glucose of known concentration. The apparent concentration of glucose in the filtrates of these solutions was determined.

Added glucose (µg./ml.)	4.0	6.0	8.0	10.0
Estimated glucose (µg./ml.)	4.02	6.01	8.12	10.10
Percent. recovery	100.5	100.2	101.5	101.0

produce an increase in the extinction of the reaction mixture of the same order as the negative blank. This phenomenon was not observed with subsequent batches of the resin, but the period for which the stock solution must be left to "age" has been found to vary from one day to a week. Clearly, in the application of this method, the properties of each batch of the resin must be expected to vary, but provided that the variations are of the sort which have been found in this work they do not detract from the usefulness of the method.

2. Compliance with Beer's Law

Fig. 23 depicts the relationship between the extinction at 625 nm and the concentration of glucose in the sample. The relationship is not linear at high concentrations of glucose, but at concentrations below 20 µg./ml. Beer's law is obeyed. All estimations of glucose with this method have been made on samples whose concentrations of glucose were not greater than 15 µg./ml. and with standard solutions containing 5, 10 and 15 µg./ml. or 4, 8 and 12 µg./ml.

3. The Effect of Deproteinisation

The apparent concentrations of glucose were determined in the filtrates of standard solutions of glucose in which zinc hydroxide had been precipitated by the procedure which was described on p. 75. The results of this experiment are presented in Table 6. No significant discrepancy was detected between the apparent and the theoretical concentrations of glucose.

4. The Accuracy of the Estimates of Glucose Concentration

The mean and the standard deviation from the mean of estimates of the concentration of glucose in the filtrates, which were obtained when samples

TABLE 7

THE ACCURACY OF THE ESTIMATION OF GLUCOSE CONCENTRATION WITH GUM GUAIACUM RESIN AS CHROMOGEN

Four estimates were made of the concentration of glucose in a deproteinised solution. The mean and standard deviation of these estimates are shown for eleven instances. The mean standard deviation is 0.06 µg./ml. The percent. error is also given.

Mean estimate (µg./ml.)	Standard deviation (µg./ml.)	Percent. error
5.13	0.03	0.53
5.14	0.02	0.37
5.15	0.05	1.03
7.46	0.02	0.27
7.51	0.08	1.09
7.97	0.09	1.09
9.99	0.05	0.45
11.88	0.08	0.69
12.01	0.10	0.83
15.03	0.05	0.34
15.36	0.10	0.65

TABLE 8

EFFECT OF RAFFINOSE ON THE ESTIMATION OF GLUCOSE WITH GUM GUAIACUM RESIN AS CHROMOGEN

The apparent concentration of glucose was measured in nine solutions containing known amounts of glucose and raffinose.

Added glucose (μg./ml.)	Estimated glucose (μg./ml.) in solutions containing: 0.5 mg. raffinose/ml.	1.0 mg. raffinose/ml.	1.5 mg. raffinose/ml.
5.0	4.99	4.97	5.02
10.0	10.05	10.01	10.05
15.0	14.98	14.92	14.98

of perfusate and infusate were deproteinised, are given in Table 7. All batches of gum guaiacum resin were represented in the reagents with which these results were obtained. The standard deviation of the estimate of a concentration is independent of the magnitude of the concentration and has a mean value of 0.06 µg./ml.

5. The Effect of Raffinose on Glucose Estimation

Inulin, which had been used in the determination of the volume of extracellular water, was replaced by raffinose, for reasons which will be discussed in the following Section, at the stage in this investigation at which gum guaiacum resin was substituted for o-tolidine as the chromogen in the estimation of glucose. Table 8 shows the results of the estimation of the apparent concentration of glucose in solutions which also contained raffinose at concentrations at least as great as those which would be present when a sample of the perfusate was estimated for glucose. It can be concluded from the table that raffinose, at the concentrations which were studied, does not affect the estimation of glucose.

Discussion

The two methods of glucose analysis, which have been described, gave accurate estimates of glucose concentrations. With both methods, the standard deviations of the estimates were independent of the magnitude of the concentration and were not significantly different at the 5% level of probability. The variations in the estimates can be ascribed largely to errors in the measurement of the per cent transmittance from traces on recorder paper which is graduated in units of 0.5% and to the error from

Table 9

CONDITIONS FOR THE DETERMINATION OF GLUCOSE UTILISATION WITH AN ERROR OF LESS THAN 10%

In a steady state, glucose utilisation is the product of the rate of infusion and the concentration difference between infusate and perfusate glucose. The infusion rate was taken to be 30.0 ± 0.6 ml./hour. The standard deviation of the estimate of the difference in concentration of two samples of equal dilution was taken to be 0.1 µg./ml. The standard deviation of an estimate of utilisation and, in parentheses the percent. error, are given for several rates of utilisation and concentration of infusate glucose.

Glucose utilisation (mg./hour)	Infusate glucose and dilution for analysis			
	10 mg./100 ml. 1:10	25 1:25	50 1:50	100 1:100
1	1 ± 0.035 (3.5%)	1 ± 0.074 (7.4%)	1 ± 0.144 (14.4%)	1 ± 0.286 (28.6%)
2	2 ± 0.049 (2.5%)	2 ± 0.082 (4.1%)	2 ± 0.148 (7.4%)	2 ± 0.288 (14.4%)
3	--	3 ± 0.093 (3.1%)	3 ± 0.155 (5.2%)	3 ± 0.291 (9.7%)
4	--	--	4 ± 0.163 (4.1%)	4 ± 0.296 (7.4%)
5	--	--	--	5 ± 0.302 (6.0%)

irregular changes in the baseline trace which are not nullified by making duplicate estimates in a series with mirror symmetry.

The higher value for the standard deviation, 0.07 µg./ml., was obtained with the method in which o-tolidine was the chromogen. From this figure, the standard deviation of the estimate of the difference between the concentrations of two samples was calculated to be 0.10 µg./ml. When samples of the infusate and the perfusate were found, after equal dilution, to differ by more than 1.0 µg./ml. in their content of glucose, the error in the estimate of the difference between the concentration of infusate and perfusate glucose was, therefore, less than 10%. Table 9 shows the minimum rate of glucose utilisation which could be measured with an error of less than 10% when the samples of the infusate and the perfusate are diluted between 10- and 200- fold and when it is assumed that a 2% error is incurred in the measurement of an infusion rate of 0.5 ml./min. An error of less than 10% in the determination of glucose utilisation will ensure that biological variation is the determinant of the overall variation in a series of experiments.

The accuracy of the estimation of glucose will be most likely to limit the useful study of the utilisation of glucose in a steady state when the uptake of glucose is maximal, at high concentrations of perfusate glucose, in the absence of insulin. This rate of utilisation by a heart which weighs 0.8 g. when wet would be approximately 2.5 mg./hour, according to the results of Bleehen and Fisher (1954) or approximately 10 mg./hour, according to the results of Morgan, Henderson et al.(1961). From the former value and the data of Table 9, the measurement of glucose utilisation in the absence of

insulin, by the technique which has been developed for this investigation, would be expected to be limited to experiments in which the concentration of perfusate glucose was less than 100 mg./100 ml. On the other hand, the results of Morgan et al. suggest that no significant limitation to the experimental conditions should occur. It is, of course, possible to extend the range of concentration by using a lower rate of infusion than 0.5 ml./min. provided that the period of perfusion within which a steady state is established remains acceptable.

Figure 24.

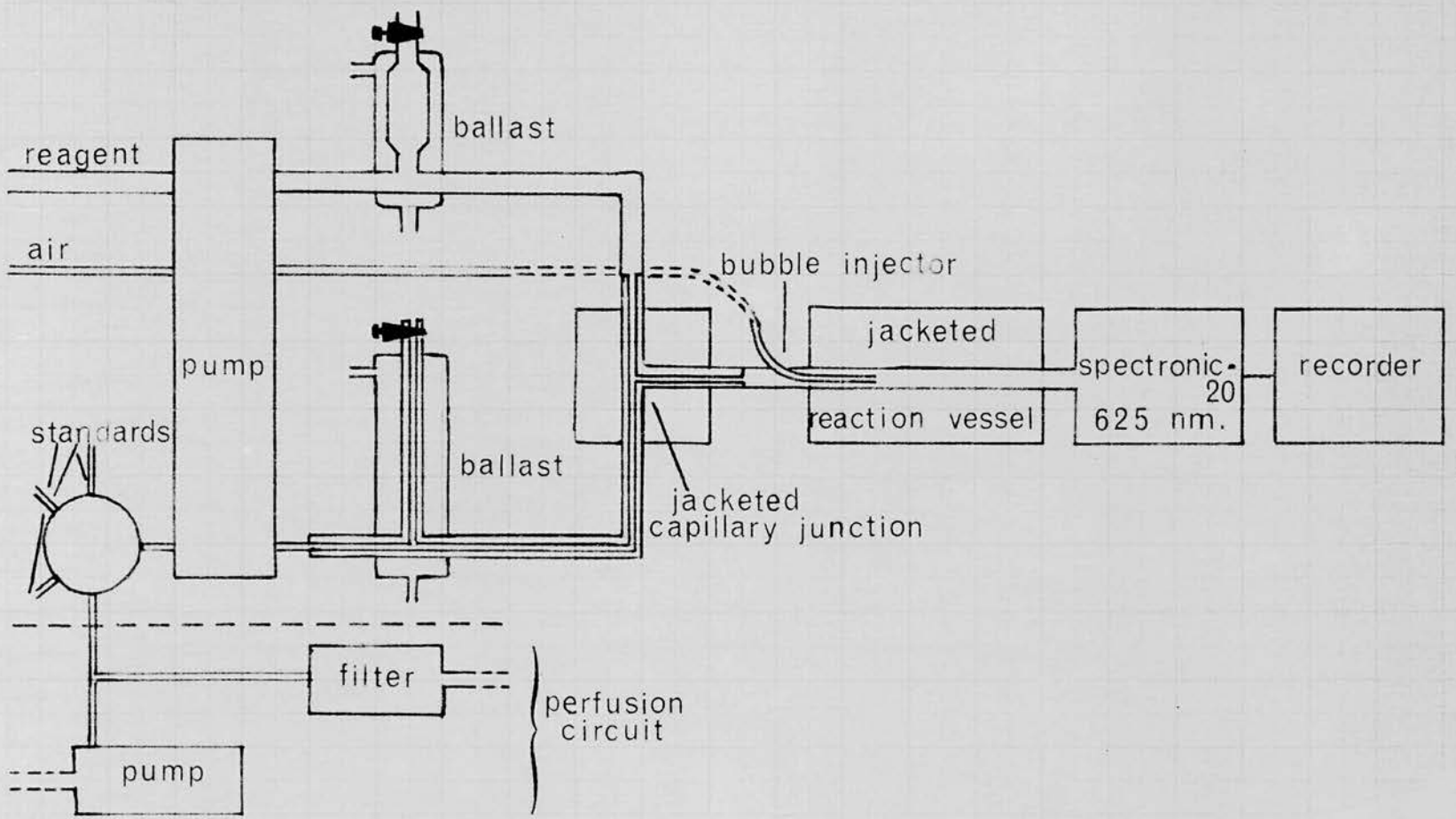


Diagram of the Monitoring Apparatus

CHAPTER THREE

THE TIME-COURSE OF THE CONCENTRATION OF PERFUSATE GLUCOSE

A SYSTEM FOR THE CONTINUOUS ESTIMATION OF PERFUSATE GLUCOSE

A system which would produce a continuous record of the concentration of perfusate glucose was sought for the sake of an early recognition of a steady state and for its potential use in the examination of the effects on a steady state of glucose utilisation which might result from the introduction of a second metabolite, a hormone or drug into the perfusate.

An attempt was made to construct an apparatus for this purpose on the principle of the Autoanalyser. The apparatus is illustrated in Fig. 24. In essence, approximately 0.2 ml./min. of the recirculated perfusate was mixed with approximately 4.0 ml./min. of the reagent in which o-tolidine was the chromogen. The reaction mixture was segmented with air and, approximately 20 seconds after its formation, it entered a flow cell. The per cent transmittance of light at 625 nm through the reaction mixture was measured and recorded. Only approximate values for the rates of pumping and the reaction time are given because the peristaltic pump was regulated in a similar manner to that which was used to control the rate of the infusion and withdrawl of perfusate. In this case, a Velodyne motor drove the pump and could be adjusted to vary the flow of the reaction mixture and

therefore the reaction time. Small changes could be made in the speed of the motor so that by altering the reaction time, the reaction mixture had a suitable extinction when it entered the flow-cell.

The initial concentration of glucose in the reaction mixture can be as much as 30 times greater than the concentration in the reaction mixture which is used in the methods for glucose estimation with the Autoanalyser. Development of colour is very rapid and its measurement presents several difficulties. A peristaltic pump generates a pulsatile flow of sample and reagent. When a small pulsatile flow of a relatively concentrated solution of glucose is introduced into a considerably greater flow of reagent, their irregular and incomplete mixing results in variations in the extinction of the solution which enters the flow-cell, even when the stream has been segmented with air and passed through a mixing coil. Consequently the trace on the recorder is marred by "noise".

The pulsatile flow of the reagent and the sample of perfusate was eliminated before their junction by placing a pressure ballast between the pump and a section of tubing of narrow bore which provided a resistance to flow. For the stream of reagent, the ballast comprised a T-piece whose side-arm opened into a chamber with a capacity of 25 ml. The flow from the narrow bore tubing was regular because the surges from the pump were damped by their compressing the air in the side-arm of the T-piece. A tap allowed air to be introduced into the chamber in order to control the level to which the reagent rose in the side-arm. The chamber and T-piece were water-jacketed, because this was found to improve the quality of the trace on the recorder. A similar water-jacketed T-piece damped the surge in the flow of

sample, but, in this case, the T-piece was made of capillary tubing in order to reduce the volume of perfusate which it could hold. The side-arm was extended with capillary tubing to be 30 cm. long. These devices eliminated all visible evidence of pulsatile flow, yet the beneficial results of insulating the ballasts could be readily detected in the reduced "noise" on the trace without the insulations having any apparent effect on the nature of the flow.

The mixing of the sample with the reagent was best achieved, again as judged by the level of "noise" on the recorder, when the two streams were brought together in the cross-arm of a water-jacketed capillary T-piece and the mixture led out through the side-arm. This procedure was most effective when the distortion of the capillary at the junction was kept to a minimum in the construction of the T-piece.

The introduction of the air, which segments the reaction mixture, was also complicated by a tendency for its flow to be pulsatile. When the air entered the reaction mixture at a T-piece of any bore, the solution entered the arm through which the air was injected after each bubble had passed into the stream. This resulted in a pulsatile flow of the reaction mixture. When the air was introduced through Tygon tubing, which had been inserted in another Tygon tube through a closely fitting hole in its wall so that it lay along the centre of the tube, the bubbles formed in the centre of the stream of reaction mixture, which had passed around the inserted tube. With this device, the reaction mixture was segmented without the flow becoming pulsatile. The segmented stream was passed through a water-jacketed glass tube before it entered a flow-cell. All the water-jacketed components of this

apparatus were maintained at 25°C by water which was pumped through the jackets by a Circotherm.

The flow-cell had a light path of 6 mm. and was of the type which was used in the Autoanalyser. It was modified, without affecting the principle of its operation, so that it could be held in a rubber bung and placed in the position of the tube-holder of a Spectronic 20 colorimeter. The per cent transmittance of light at 625 nm through the cell was recorded on a Bausch and Lomb VOM 5 recorder.

Perfusate was sampled through a T-piece which was placed between the perfusate pump and the filter (Fig. 5). The sample passed through one of 4 inlets to a polythene tap which had one outlet to the monitoring apparatus. Three standard solutions of glucose and the perfusate were aspirated in turn to produce a record from which the concentration of perfusate glucose could be estimated at any time.

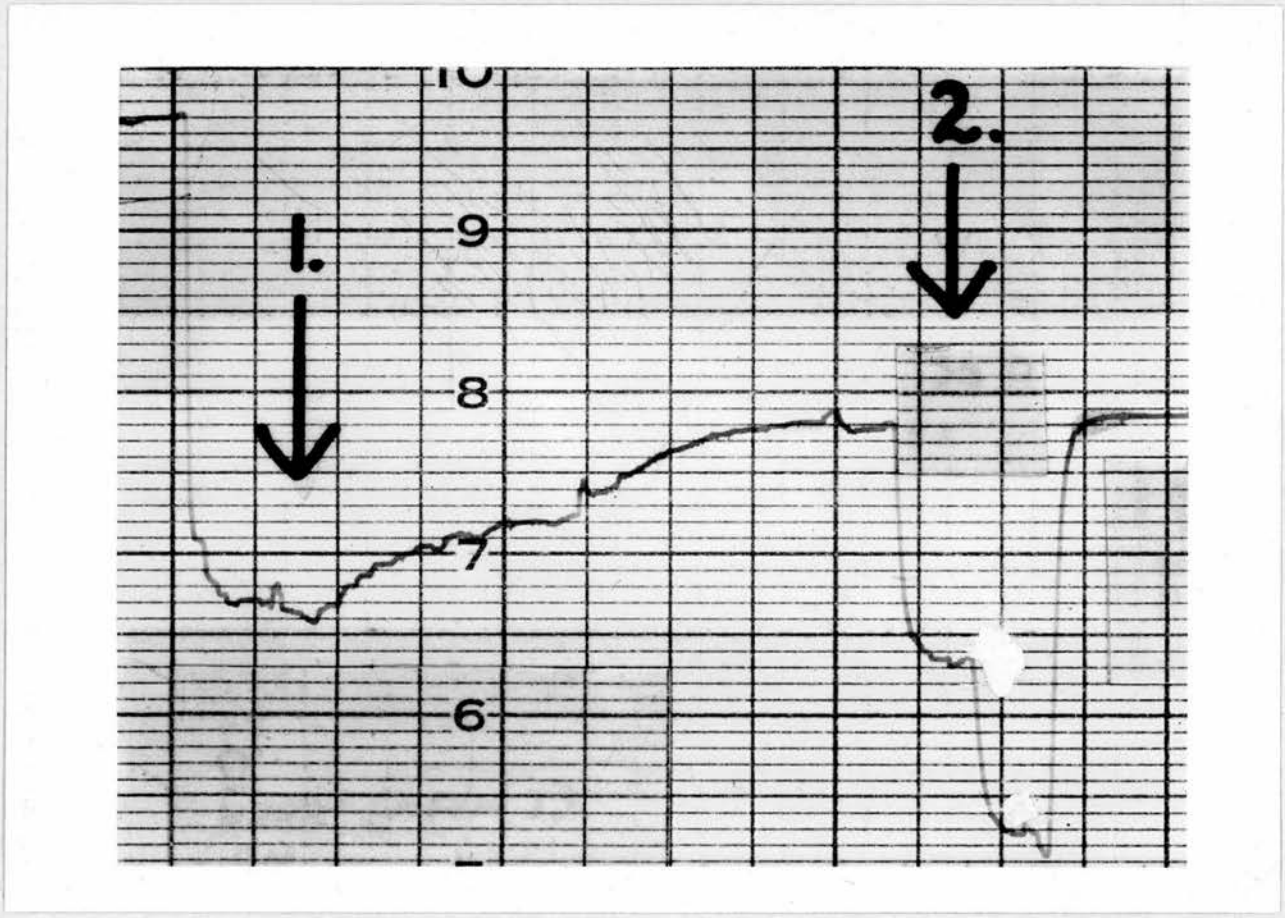
Results

In the initial attempts to measure the concentration of glucose in this apparatus, Triton X-100 was not included in the reagent, in which o-tolidine was the chromogen. The trace, which resulted from the aspiration of a standard solution of glucose, commonly showed a progressive decrease in the percentage transmission. When water was aspirated in place of the glucose solution, the percentage transmission remained constant but at a level which was reduced after each period when the solution of glucose was aspirated. It was concluded that the product of the oxidation of o-tolidine was adsorbed on the surface of the flow-cell. The effect of the inclusion of Triton X-100

Fig. 25

TIME-COURSE OF PERFUSATE GLUCOSE CONCENTRATION

A trace from the monitoring apparatus



At arrow 1. cardiac perfusion began. At 2., three standard solutions were estimated, of which the most dilute was at almost the same glucose concentration as the perfusate.

was examined because Dahlqvist (1964) found that this detergent improved the stability of the colour which is produced when o-dianisidine is oxidised. With this modification to the reagent, the percentage transmission remained constant during the aspiration of a standard solution of glucose. No influence of Triton X-100 on the estimation of glucose in the Autoanalyser has been detected, but, because the initial concentration of glucose in the reaction mixture which was formed in the monitoring apparatus could be 30 times greater than that in the Auto-analyser, a higher concentration of the coloured product might have existed in the boundary layer of the reaction mixture which passed through the cell of the monitoring apparatus.

Despite the precautions which were taken in the construction of the apparatus, "noise" could not be completely eliminated from the trace on the recorder. Consequently the accuracy with which the concentration of perfusate glucose could be measured was limited, but the traces did suggest that a constant concentration of glucose was established. Such a trace, which was obtained when a heart was perfused in the presence of insulin is shown in Fig. 25. The concentrations of the standard solutions of glucose which calibrated this record were 0.35, 0.70 and 1.05 mg./ml., from which the concentration of glucose in the apparent steady state was estimated to be 0.33 mg./ml. The rate of utilisation of glucose was calculated to be 63 mg./g. dry weight/hour.

The use of the monitoring apparatus was limited to the demonstration, in preliminary experiments, of the feasibility of attaining a steady state. When the record indicated that a constant concentration of perfusate glucose had been established, the perfusate which was withdrawn from the reservoir

TABLE 10

CONSTANCY OF PERFUSATE GLUCOSE CONCENTRATION WHEN HEARTS ARE PERFUSED WITH INSULIN

Three successive fractions of perfusate were collected for 10 minute periods after one hour's perfusion. Triplicated estimates were made of the glucose concentration of each fraction. The constancy of the concentration in the 30 minute period was examined by an analysis of the variance of the three sets of estimates. The probability of variations between the fractions being random is given for five experiments.

	Fraction 1	Fraction 2	Fraction 3	Overall	P
Mean and standard deviation of glucose concentration (μg./ml.)	5.53 ± 0.05	5.53 ± 0.04	5.64 ± 0.01	5.57 ± 0.06	$0.05 > P > 0.01$
	4.93 ± 0.03	4.93 ± 0.03	4.95 ± 0.03	4.94 ± 0.03	$P > 0.05$
	7.20 ± 0.04	7.24 ± 0.02	7.24 ± 0.05	7.23 ± 0.04	$P > 0.05$
	5.51 ± 0.03	5.37 ± 0.04	5.36 ± 0.05	5.41 ± 0.08	$0.01 > P$
	6.29 ± 0.04	6.43 ± 0.07	6.28 ± 0.03	6.33 ± 0.08	$0.05 > P > 0.01$

to maintain a constant volume of recirculated perfusate was collected for three successive periods of 10 minutes. Accurate estimates of the concentration of glucose in these fractions were then made in the Autoanalyser. Table 10 presents the results of several experiments in which the constancy of the concentration of the perfusate glucose was tested in this manner. Within the limits of the accuracy of the analyses, a constant concentration was maintained during the period of 30 minutes in two instances. In three other cases, an analysis of variance indicates a significant difference between fractions. These differences are small and probably reflect the difficulty of maintaining a preparation for 30 minutes without any change in the properties of the heart.

The difficulties, which had been experienced in developing the monitoring apparatus to provide a qualitative indication of the approach of the concentration of perfusate glucose to a constant level, did not encourage further development. Nevertheless, considerable improvements in the performance of the apparatus could probably now be achieved.

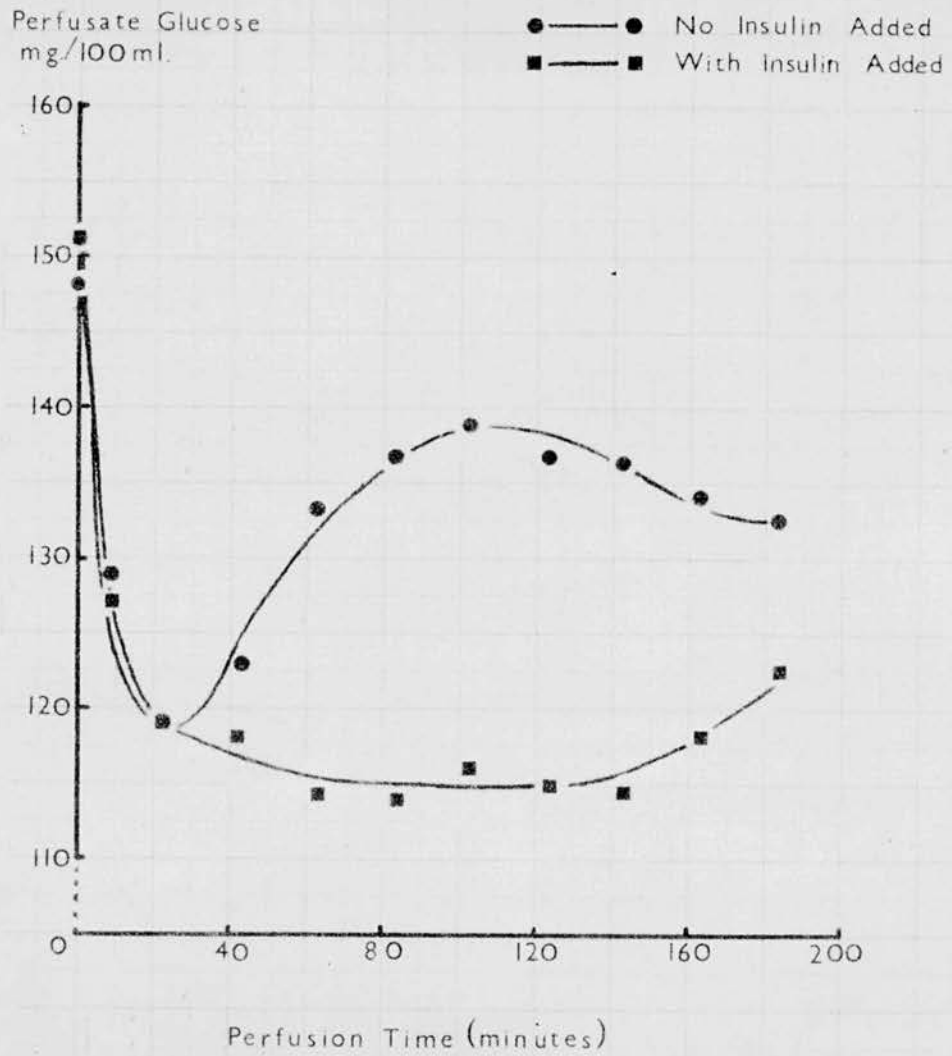
THE TIME-COURSE OF PERFUSATE GLUCOSE CONCENTRATION FROM PERIODIC ANALYSES

Although the monitoring of the concentration of perfusate glucose did not provide accurate measurements, the collection and the analysis of fractions of the perfusate which was continuously withdrawn from the perfusion reservoir did allow the precise estimation of the concentration of glucose in these fractions. The collection of fractions at regular intervals throughout an experiment enabled changes in the concentration of glucose to

Figure 26.

TIME-COURSE OF PERFUSATE GLUCOSE CONCENTRATION

Effect of Insulin at high concentrations of Glucose

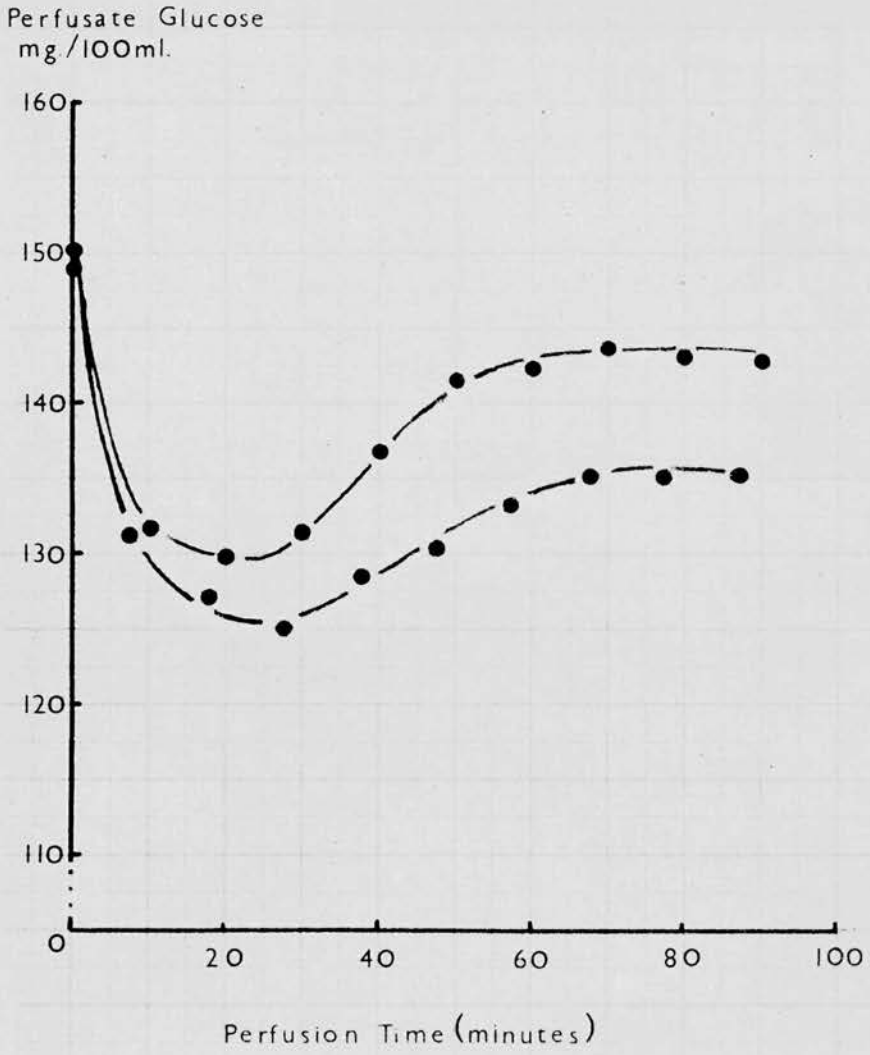


Fractions were collected for 5 min. periods and their glucose concentration plotted against the time at the mid-point of the period. The lines are drawn through points calculated from the fourth-order polynomial which best fits the data.

Figure 27.

TIME--COURSE OF PERFUSATE GLUCOSE CONCENTRATION

Hearts perfused at high concentrations of Glucose without exogenous Insulin.



Two experiments are illustrated. Fractions were collected for 4 min. periods and their glucose concentration plotted against the time at the mid-point of the period. The lines are drawn through points calculated from the fourth-order polynomial equation which best fits the data.

be followed. However, while a successful monitoring system would allow the instantaneous concentration of glucose to be measured, the concentration in a fraction necessarily reflected the mean concentration of the recirculated perfusate during the period of collection. Fractions were collected for 4 or 5 minute periods to provide 2.0 to 2.5 ml. for analysis. Variations in the rate of change of the concentration of perfusate glucose in the period of collection will clearly determine the confidence with which the concentration in the fraction can be attributed to the concentration in the perfusate at the mid-point of the collection. In a steady state, no error can result from this procedure.

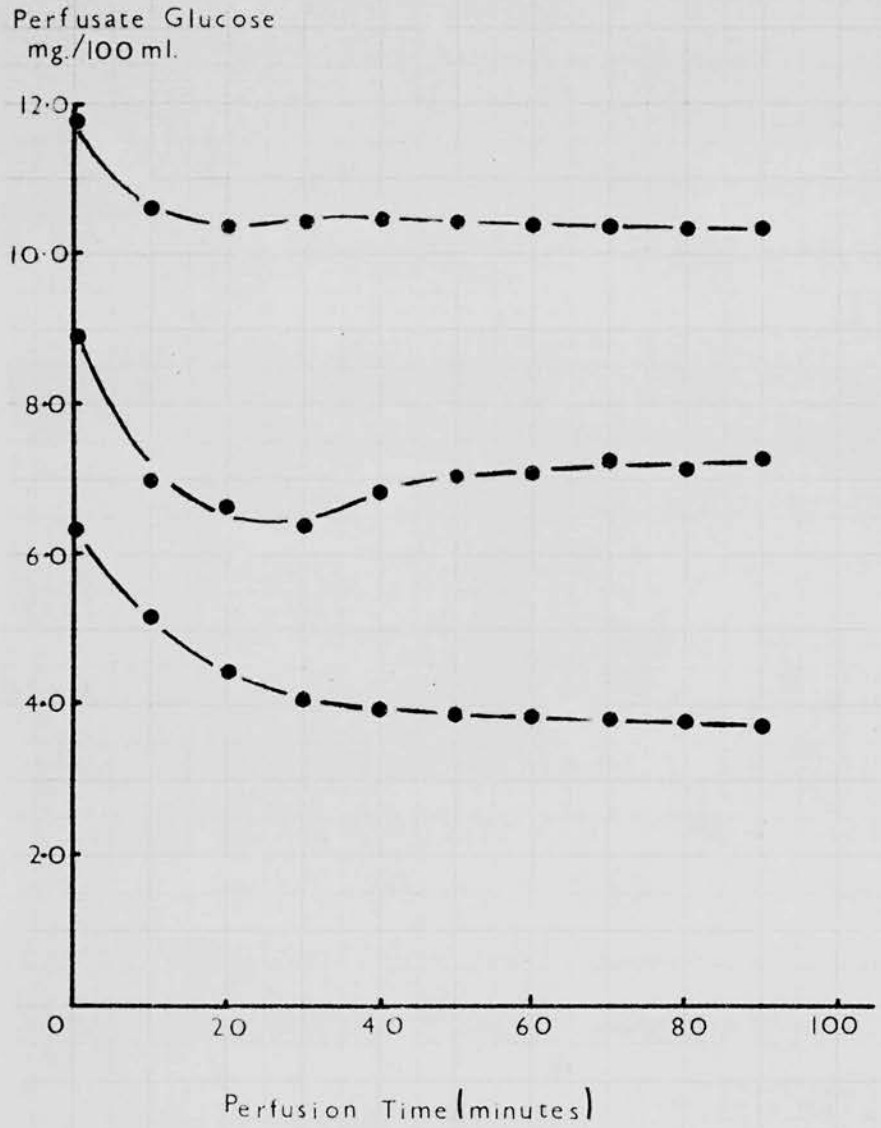
In Fig. 26 the concentration of perfusate glucose is plotted against the time of perfusion in two experiments, which were prolonged for 3 hours. In one case, insulin was present in the perfusate at 100 mU/ml. The experiments were timed from the introduction of the heart into the perfusion apparatus. As was the case in all the experiments to be described, the perfusate to which the heart was then exposed was identical with the infusate. The initial concentration of the perfusate glucose was therefore taken to be that of the infusate. A minimum volume of recirculated perfusate was established within 4 minutes and a fraction was collected from the end of the fifth to the end of the tenth minute of perfusion. Subsequent fractions were also collected for 5 minute periods so that the mid-points of the collections were at 20 minute intervals.

More detailed plots of the time-course of the concentration of glucose, when hearts were perfused without insulin and the infusate contained 150 mg. of glucose/100 ml., are shown in Fig. 27. The procedure which was adopted

Figure 28.

TIME-COURSE OF PERFUSATE GLUCOSE CONCENTRATION

Hearts perfused at low concentrations of Glucose without exogenous Insulin



Three experiments are illustrated. Fractions were collected for 4 min. periods and their glucose concentration plotted against the time at the mid-point of the period. The lines are drawn through points calculated from the fourth-order polynomial equation which best fits the data.

in the experiment depicted by the upper curve differed from that which was described above in that fractions were collected for 4 minutes centred about 10 minute intervals from the initiation of perfusion. In all other experiments, in which the concentration of glucose was less than 150 mg./100 ml., a standard procedure was established for the periodic collection of fractions. These experiments were timed from the moment of the existence of a constant minimum volume of recirculated perfusate. A first fraction was collected from the perfusate which was withdrawn from the reservoir immediately before and for 2 minutes after the establishment of a minimum volume. Subsequent fractions were collected for 4 minutes centred about 10 minute intervals from zero time. This procedure permits the changes in the concentration of glucose to be studied under constant conditions from the first practicable moment after the beginning of the perfusion.

The pattern in Figs. 26 and 27 of the time-courses of perfusate glucose when hearts were perfused without insulin is typical of those observed in all but one of 17 experiments in which the concentration of perfusate glucose was greater than 20 mg./100 ml. After a decline in the concentration of perfusate glucose in the first 30 minutes of perfusion, the concentration rose and tended to become constant after 90 minutes. However, when the concentration of perfusate glucose was less than 20 mg./100 ml., the pattern of the time-course of the concentration in 27 experiments showed a less marked minimum or none at all. This was particularly evident at the lowest steady-state concentration of glucose - 4 mg./100 ml. (Fig. 28).

In the presence of insulin, the time-course of the perfusate glucose in 25 experiments was invariably of the sort shown in Fig. 26 which, in the

TABLE 11

CONSTANCY OF PERFUSATE GLUCOSE CONCENTRATION WHEN HEARTS ARE PERFUSED WITHOUT INSULIN

Three successive fractions of perfusate were collected for 5 minute periods after 75 min. of perfusion. Duplicated estimates were made of the glucose concentration of each fraction. The constancy of the glucose concentration in the 15 min. period was examined by an analysis of the variance of the three sets of estimates. The probability of the variations between the fractions being random is given for six experiments.

	Fraction 1	Fraction 2	Fraction 3	Overall	P
Mean and standard deviation of glucose concentration (μg./ml.)	6.84	6.77	6.92	6.84 ± 0.07	$P > 0.05$
	9.09	9.17	9.29	9.18 ± 0.10	$P > 0.05$
	7.57	7.50	7.54	7.54 ± 0.04	$P > 0.05$
	12.32	12.27	12.29	12.29 ± 0.02	$P > 0.05$
	8.36	8.25	8.18	8.26 ± 0.09	$P > 0.05$
	10.36	10.29	10.27	10.31 ± 0.05	$0.05 > P > 0.01$

first 2 hours, might be expected to be described by a simple exponential equation. The number of observations which were made when the concentration of glucose was changing rapidly was too small to permit an accurate determination of a rate constant. From an inspection of the data from several experiments, the half-time for the approach to a constant level of perfusate glucose was estimated to range from 7 to 10 minutes, which compares well with the theoretical half-time of 8.3 minutes for a system with a constant volume of 6 ml. and an infusion rate of 0.5 ml./min. (p.23).

The Effect of Anti-Insulin Serum on the Time-Course of Perfusate Glucose

Figs. 26, 27 and 28 indicate that, whether or not insulin was included, a constant concentration of glucose existed in the perfusate within 90 minutes of perfusion. However, at high concentrations of glucose without added insulin, the period of stability was short, when an experiment was terminated after 90 minutes. In prolonged experiments, the concentration of glucose always decreased before 2 hours of perfusion were completed and, often, this decrease occurred after a period of stability of less than 20 minutes. The estimation of the glucose concentration of three successive samples in the final 15 minutes of a 90 minute perfusion provided additional evidence of the stability of the concentration in this period (Table 11). Nevertheless, the condition of a heart, which might be analysed to determine the intracellular concentration of glucose after perfusion for 90 minutes in the absence of insulin, might be in doubt in view of the likelihood of an imminent loss of metabolic stability.

The time-course of the concentration of perfusate glucose, when a heart

was perfused without insulin, can be explained by an initial decrease in the utilisation of glucose, which is in part independent of the changes in the concentration of glucose. Because the most reasonable cause of such a decrease would be the loss of endogenous insulin from the heart, the effect of anti-insulin serum on the time-course of perfusate glucose was investigated, in the hope that the neutralisation of endogenous insulin would allow the more rapid attainment of a constant concentration of perfusate glucose.

Anti-insulin serum was generously donated by Dr. B. Hurne of the Wellcome Research Laboratories, Beckenham. The serum had been prepared from the blood of guinea-pigs which were immunised with repeated doses of ox and pig insulin. One ml. of the serum was reported to be capable of neutralising at least one international unit of ox insulin.

0.05, 0.1 or 0.2 ml. of the serum was included in 15 ml. of perfusate with which a heart was perfused in closed circuit for 5 minutes before the infusion and withdrawal of perfusate was begun. In these circumstances, the heart was perfused for 8 or 9 minutes before a minimum volume of recirculated perfusate was established and before the first sample of perfusate was collected. No serum was present in the infusate.

This treatment had no effect on the time-course of the concentration of the perfusate glucose during a further 90 minutes of perfusion, in 31 experiments, other than what would be expected of the delay in obtaining the first and subsequent estimates of the concentration of perfusate glucose. The characteristics of the time-course were identical with those which were observed when an untreated heart was perfused, whether at a high or at a low concentration of perfusate glucose.

To test the possibility that the anti-insulin was adsorbed on the surfaces of the perfusion apparatus and that this could be reduced by the inclusion of more protein in the perfusate, hearts were pre-perfused for 5 minutes with 15 ml. of perfusate which contained 0.05 ml. of the anti-insulin serum and bovine serum albumin at 0.1 g./100 ml. The concentration of infusate glucose was 50 or 100 mg./100 ml. during the subsequent 90 minutes of perfusion, and the pattern of the time-courses conformed to that observed at high concentration of perfusate glucose when an untreated heart was perfused.

The Effect of the Nature of the Filter on the Time-Course of Perfusate Glucose

The filtration of perfusate through a fine sintered glass disc was shown by Zachariah (1961) to have a deleterious effect on the permeability of the perfused rat heart unless bovine serum albumin was included in the perfusate. In the absence of the protein, a decrease in the permeability of the heart to L-arabinose in the first 45 minutes of perfusion was followed by a considerable rise in permeability during the subsequent 30 minutes of perfusion. In the presence of the protein, the initial decline in permeability was followed by a period of 45 minutes during which the permeability was stable.

The sintered glass filter in the apparatus of Zachariah was twice as large in both diameter and thickness as that used in this work. If it were assumed that the deleterious effect of the filter on the permeability of the heart was caused by adsorption on the filter of some substance, which is

eluted from the heart during perfusion, the smaller surface area of the filter used in this work might be less effective in this respect. Certainly, the time-course of perfusate glucose in the absence of insulin suggests that the properties of the hearts became stable after perfusion for 30 minutes. No difference was detected in the character of the time-course when the perfusate was filtered by sintered glass or Millipore. The latter filter was preferred because the sintered glass disc was liable to break and to cause some experiments to be fruitless.

The mathematical analysis of the time-course of perfusate glucose, which permits the utilisation of glucose to be determined during the approach to a constant concentration of perfusate glucose, provides a clearer indication of the stability of the properties of the heart. This analysis is presented in the following chapter.

CHAPTER FOUR

THE TIME-COURSE OF GLUCOSE UTILISATION

INTRODUCTION

The principle of the system for cardiac perfusion, which was used in this work, was illustrated in Fig. 2, p. 21. Infusate is introduced into a volume of recirculated perfusate which is kept constant by withdrawing perfusate at a rate equal to that of infusion. An analysis of the time-course of perfusate glucose concentration in this system is more complete if the restriction on the movement of glucose from the perfusate into the interstitial water is taken into account.

Let: P ml. be the volume of recirculated perfusate,

Q ml. be the volume of interstitial water,

x mg./ml. be the concentration of perfusate glucose,

c mg./ml. be the concentration of interstitial glucose,

a mg./ml. be the concentration of infusate glucose,

i ml./min. be the rate of infusion,

h mg./min. be the rate of glucose utilisation,

r $min.^{-1}$ be the rate constant for the trans-capillary movement of glucose,

t min. be the time of perfusion.

The rate of change of the amount of glucose in the perfusate is given by:-

$$P.\frac{dx}{dt} = ia + Qrc - ix - Qrx \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

and the rate of change of the amount of glucose in the interstitial water is given by:-

$$Q.\frac{dc}{dt} = Qrx - Qrc - h \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

From (1):

$$Qrc = P.\frac{dx}{dt} + ix + Qrx - ia \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

and differentiating (3):

$$Qr.\frac{dc}{dt} = P.\frac{d^2x}{dt^2} + i.\frac{dx}{dt} + Qr.\frac{dx}{dt} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (4)$$

Substituting from (3) and (4) in (2) and rearranging gives:

$$h = i(a - x) - (\frac{i}{r} + P + Q)\frac{dx}{dt} - \frac{P}{r}.\frac{d^2x}{dt^2} \quad .. \quad .. \quad .. \quad .. \quad (5)$$

As is shown below, the values of P, Q and r are such that equation (5) approximates closely to:

$$h = i(a - x) - P.\frac{dx}{dt} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

It may be noted that in equation (6), h strictly defines the rate of loss of glucose from the perfusate to the interstitial water. The statement that equation (5) in practice approximates to equation (6) therefore amounts to the claim that in the experimental conditions the net rate of permeation of the myocardial cells was indistinguishable from the net rate of movement of glucose into the interstitial water.

The difficulties of measuring the volume of recirculated perfusate (P) were discussed on p. 44. Variations in the volume occurred because of the impossibility of reproducibly collapsing the poly-vinyl finger around the heart in each experiment. The most probable value for the volume was 6.0 ml. and this figure has been used for all calculations of the time-course of glucose utilisation. The volume of interstitial water (Q) must be little less than the volume of the extracellular water of the blotted rat heart. For this calculation, Q was taken to be 0.3 ml. (Fisher and Young, 1961). A minimum value for the rate constant (r) for the movement of glucose across the capillary walls can be estimated from equation (2) which becomes:

$$Qrx - Qrc - h = 0 \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (7)$$

when a steady state is reached. Thus:

$$x - c = \frac{h}{Qr}$$

Since a perfusate glucose concentration (x) of 0.05 mg/ml. was found to sustain a rate of glucose utilisation (h) of 0.05 mg./min., r must be

greater than 3.33 min.$^{-1}$. Substituting these values for P,Q and r in equation (5) and taking the infusion rate (i) to be 0.5 ml./min.:

$$h = i(a - x) - 6.45 \cdot \frac{dx}{dt} - 1.8 \frac{d^2x}{dt^2}$$

Substituting in equation (6) gives:

$$h = i(a - x) - 6.0 \frac{dx}{dt}$$

As in the examples in a subsequent table (Table 15), the term P.dx/dt contributes less than 50% of an estimate of utilisation, even when the concentration of perfusate glucose is changing rapidly at the beginning of perfusion. In 6 instances which were examined, the term $P/r. \ d^2x/dt^2$ constituted less than 3% of an estimate of glucose utilization when the rate of change of glucose concentration altered most rapidly at the beginning of perfusion. At this time of perfusion, dx/dt and d^2x/dt^2 are of opposite sign so that the error which is introduced by using the approximate form given by equation (6) is in part nullified. In all these calculations the error has been exaggerated by taking a minimum value for r. Thus, the utilisation can be determined at any time from a knowledge of the infusion rate, the concentration of infusate glucose, the concentration of perfusate glucose and its rate of change, and the volume of recirculating perfusate at that time.

The Determination of the Rate of Change of Perfusate Glucose Concentration

The rate of change of the concentration of perfusate glucose is given by the slope of the plot of concentration against time. An equation which related the concentration of perfusate glucose to the time of perfusion was obtained by fitting a fourth order polynomial to the experimental data. This order of polynomial was chosen to take into account the complexity of the time-course of perfusate glucose which was observed in the absence of insulin when the early minimum in the plot was followed by a maximum as the heart failed. However, whether the data were obtained from a perfusion with insulin or without insulin, the same procedure was applied.

The fourth order polynomial was fitted by the method of Aitken (1939) whereby the dependent variable is expressed not in powers, but in orthogonal polynomial functions, of the independent variable; these polynomials are the orthogonal polynomials of Tchebychef. This method requires that the values of the dependent variable are of equal weight and correspond to equispaced values of the independent variable. The coefficients of the orthogonal polynomials are calculated and, through them, the initial value and the four leading differences of the best fit values for the dependent variable are obtained. The remaining best fit values for the equispaced observations of the dependent variable are then derived from the initial value and the four leading differences. It was thus possible to compute the best fit values of the concentration of perfusate glucose which corresponded to the observations made at equispaced intervals during the course of a perfusion. The lines in Fig. 26 are drawn through the points which were calculated in this way. The coefficients of the fourth order polynomial were also calculated,

by standard practice, from the initial value and the four leading differences. Differentiation of the resultant equation enabled the rate of change of glucose concentration, at any time, to be calculated.

This procedure, which was lengthy when it was carried out with an electric calculator, was more readily conducted by a suitably programmed Olivetti Programma 101. The computation of the coefficients of the orthogonal polynomials and the best fit values for the concentration of perfusate glucose was performed by programmes which were supplied by British Olivetti Ltd., London. A further programme was written by which the four leading differences were calculated from the best fit values of glucose concentration and thence the coefficients of a fourth order polynomial. This programme is also used to differentiate the equation and to calculate the slope of the regression line at any value of time.

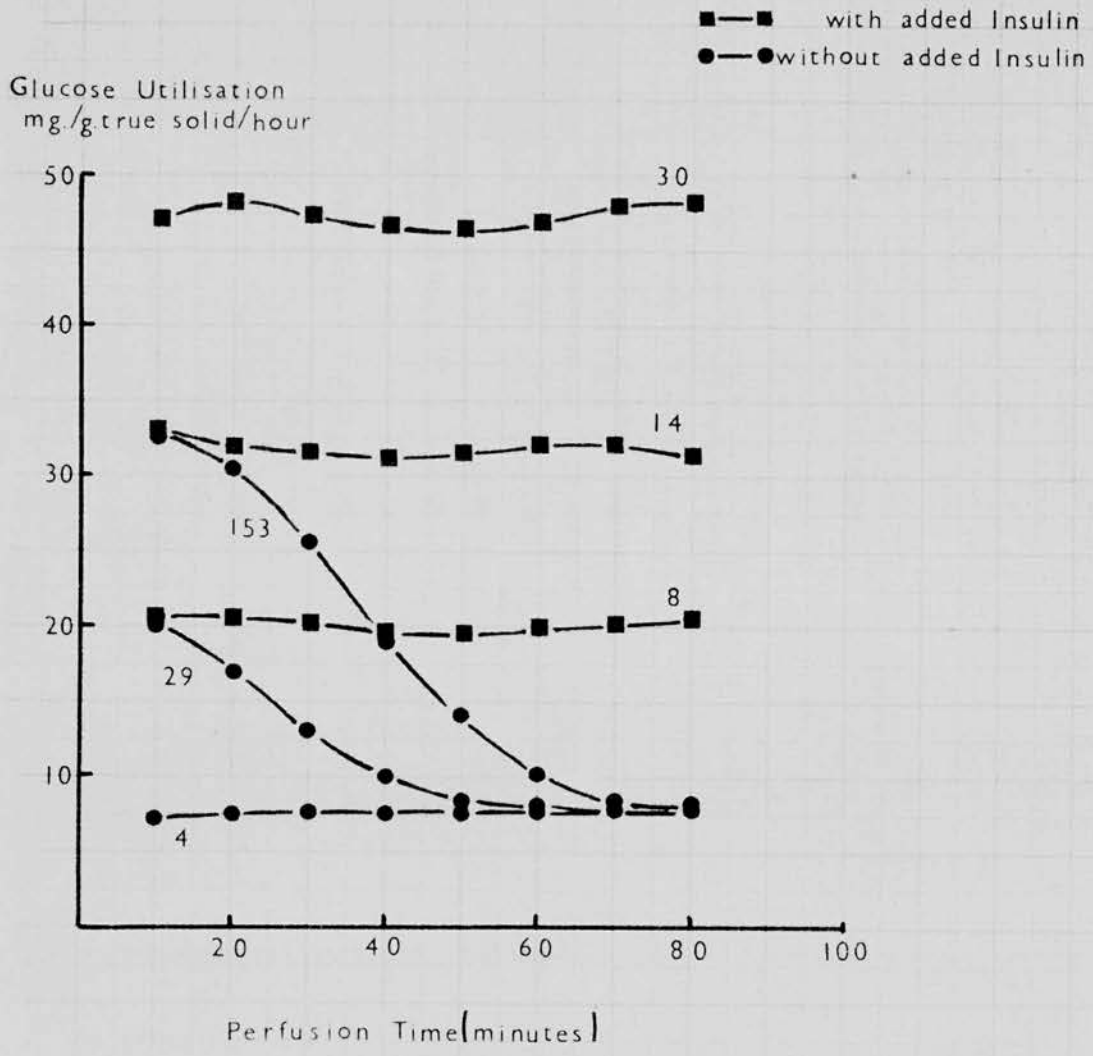
The Calculation of the Time-Course of Glucose Utilisation

Substitution of the best fit values for the concentration of perfusate glucose and the rate of change of this concentration at a particular time, together with values for the concentration of infusate glucose, the infusion rate and the volume of recirculated perfusate, in the equation (6), which is derived on p.98, enabled the utilisation of glucose, at that time, to be calculated. This computation and the correction of the result to a unit weight of tissue was also written as a programme for the Programma 101. When it was performed in this fashion, the whole computation from the curve-fitting to the calculation of utilisation took only 20 minutes. With this facility, the determination of the time-course of glucose utilisation

Figure 29.

TIME-COURSE OF GLUCOSE UTILISATION

Effect of Insulin and Glucose concentration



The tine-course of utilisation in the presence of insulin at 100 mU./ml. was determined from the best fit values for the glucose concentration and the rate of change of that concentration obtained by fitting a fourth-order polynomial to the experimental data. The number by each plot is the steady state concentration (mg. / 100 ml.) of glucose in that experiment.

became standard practice in the latter stages of this investigation, because, as will be discussed later, it conferred some advantage in the assessment of the condition of the preparation.

RESULTS

The time-courses of the utilisation of glucose in 6 experiments of 90 minutes are shown in Fig. 29. It will be noted that the initial and final values for the rate of utilisation have been omitted. These omissions were made because, in practice, the nature of the curve-fitting procedure commonly yielded anomalous values for the rate of change of glucose concentration at the beginning and end of the experiments. The examples comprise three hearts, which were perfused with insulin, and three hearts which were perfused without the addition of the hormone to the perfusate. The concentrations of glucose in the perfusates of the hearts, after 90 minutes of perfusion, are also shown.

While the utilisation of glucose in the presence of insulin remained almost constant throughout the three experiments, this constancy was only observed in the absence of insulin at a low concentration of perfusate glucose. At higher concentrations, the rate of glucose utilisation fell by as much as 75% during the first hour of perfusion. To test the conformity of this decrease in utilisation with time to a single exponential, the logarithm of the difference between any intermediate rate of utilisation and the final rate was plotted against time. The line was significantly curvilinear ($P < 0.01$) and, therefore, the relationship could not be des-

TABLE 12

THE RELATIONSHIP BETWEEN THE NUMBER OF HEARTS, FOR WHICH THE PERCENT. DECREASE IN GLUCOSE UTILISATION IN THE FIRST HOUR OF PERFUSION WAS GREATER OR LESS THAN 20%, AND THE STEADY-STATE CONCENTRATION OF GLUCOSE

Concentration of glucose (mg./100 ml.)	Number of experiments in which the decrease in utilisation was:	
	a) < 20%	b) > 20%
<10	13	5
10-20	5	4
>20	1	16

cribed by a single exponential.

The examples in Fig. 29 are illustrative of 44 experiments in which the time-course of glucose utilisation by hearts perfused without insulin was determined. In Table 12 the number of experiments in which the per cent decrease in utilization during the first hour of perfusion was greater or less than 20% is classified against the steady-state concentration of glucose in those experiments. The relative constancy with time of glucose utilisation at the lower concentration of glucose is apparent. Indeed, the average per cent decrease in utilization by the 13 hearts, whose glucose utilisation decreased by less than 20% when the concentration of perfusate glucose was less than 10 mg./100 ml., is 6.5%. Six experiments in which the steady-state concentration of glucose was less than 5 mg./100 ml. were conducted and in all the decrease in utilisation was less than 20%.

The Accuracy of the Estimates of Glucose Utilisation

Before the use of the Programma 101 made practicable the routine calculation of the time-course of utilisation, two procedures had been applied for the determination of utilisation at a constant concentration of perfusate glucose. In one, three successive fractions of the perfusate were collected, each for 10 minutes, after evidence had been obtained with the monitoring apparatus that a constant concentration of perfusate glucose existed. This technique was only used when insulin was included in the perfusate. In the other procedure, three successive fractions were collected in the final 15 minutes of a 90 minutes perfusion under experimental conditions in which the nature of the time-course of the concentration of

TABLE 13

THE ACCURACY OF ESTIMATES OF GLUCOSE UTILISATION

The standard deviation of the estimate of the difference in glucose concentration of two samples was taken to be 0.1 μg./ml. Infusate and perfusate were diluted equally. The difference between the concentration of infusate and perfusate glucose is indicated by Δ

Insulin 100 mU./ml.	Perfusate glucose mg./ml.	Δ mg./ml.	Infusion rate ml./hour	Utilisation mg./hour
+	1.149	0.366 ± 0.010	26.81 ± 0.45	9.80 ± 0.32
+	0.494	0.259 ± 0.005	24.48 ± 0.48	6.34 ± 0.17
+	0.311	0.202 ± 0.005	28.08 ± 0.61	5.67 ± 0.19
+	0.108	0.185 ± 0.001	27.46 ± 0.31	5.09 ± 0.06
+	0.055	0.085 ± 0.001	27.03 ± 0.53	2.28 ± 0.05
0	0.918	0.103 ± 0.010	29.49 ± 0.65	3.03 ± 0.30
0	0.439	0.099 ± 0.005	29.46 ± 0.53	2.91 ± 0.16
0	0.258	0.095 ± 0.0025	29.22 ± 0.39	2.77 ±0.08
0	0.105	0.046 ± 0.001	29.32 ± 0.47	1.35 ± 0.04
0	0.048	0.027 ± 0.001	29.00 ± 0.61	0.78 ±0.03

perfusate glucose was known. Although it was found by an analysis of variance that there was, in some instances, a significant difference between the concentrations of glucose in the fractions (Tables 10 and 11), these differences might have resulted from only small changes in the rate of utilisation, if, indeed, they were not the result of technical error.

In the determination of the time-course of utilisation, error was incurred in the estimates not only of the difference between the rates of addition and removal of glucose, by virtue of infusion and withdrawal, but also of the rate of change of the amount of glucose in the recirculated perfusate. The sum of these estimates gave the rate of glucose utilisation. When the concentration of perfusate glucose became so close to constancy that the rate of change of the amount of perfusate glucose contributed less than 1% to the estimate of utilisation at that time, the error of the estimate of utilisation could be treated as being determined solely by the error in the product of the rate of infusion and the difference between the concentrations of infusate and perfusate glucose. In this circumstance, the errors in the estimates of utilisation, which were obtained by the three methods, have the same origin.

Estimates of utilisation and their standard deviations are shown in Table 13. The results were obtained when hearts were perfused, with and without added insulin, at a variety of concentrations of glucose and when the utilisation was measured only at a constant concentration of glucose. The standard deviations of the estimates were calculated on the assumption that the standard deviation of the difference between the estimates of the concentration of glucose in samples of the infusate and the perfusate, after

TABLE 14

THE GOODNESS OF FIT OF A FOURTH-ORDER POLYNOMIAL TO THE TIME-COURSE OF PERFUSATE GLUCOSE CONCENTRATION

The table shows the differences between the observed and the "best-fit" value for the concentration of perfusate glucose at 10 min. intervals in the perfusion of eight hearts. The perfusate contained glucose at from 5 to 100 mg./100 ml. but no insulin. The mean difference at each time was tested for the significance of its difference from zero. No significant differences were found.

Time (min.)	Difference between observed and best-fit glucose concentration (mg./100 ml.)								Mean difference
0	-0.041	0.119	0.075	0.027	0.044	0.159	0.197	-0.104	0.060 ± 0.035
10	0.124	-0.321	-0.196	-0.070	-0.125	-0.435	-0.476	-0.067	-0.179 ± 0.078
20	-0.091	0.140	0.056	0.031	0.051	0.223	0.074	0.566	0.131 ± 0.070
30	-0.041	0.177	0.162	0.013	0.096	0.189	0.341	-0.680	0.032 ± 0.110
40	0.058	0.019	-0.005	0.037	0.026	-0.094	0.216	-0.269	0.001 ± 0.049
50	-0.030	-0.117	-0.107	-0.012	-0.194	0.166	-0.281	0.147	-0.054 ± 0.055
60	0.026	-0.145	-0.056	-0.049	0.092	-0.365	-0.342	0.869	0.004 ± 0.136
70	0.043	0.072	0.037	-0.026	0.027	-0.052	0.111	-0.625	-0.052 ± 0.084
80	-0.077	0.123	0.075	0.079	-0.014	0.351	0.315	-0.087	0.096 ± 0.058
90	0.028	-0.067	-0.041	-0.031	-0.003	-0.143	-0.155	0.114	-0.037 ± 0.031

equal dilution, was 0.10 µg./ml. The standard deviation of the infusion rate was calculated from the measurements which were made in each experiment.

At all concentrations of perfusate glucose, the error in the estimate of utilisation was less than 5%, when the hearts were perfused with insulin. When insulin was not added to the perfusate and when the concentration of perfusate glucose was of the order of 100 mg./100 ml., the error was greater and exceeded 10%.

The errors of the estimates of the time-course of utilisation are increased, when the rate of change in the amount of perfusate glucose contributes significantly to the estimate, because of the uncertainty of the value which was ascribed to the volume of recirculating perfusate. With an infusion rate of 0.45 to 0.50 ml./min. the half-time of the approach to a constant concentration of perfusate glucose was consistent with a volume of perfusate of the order of 6.0 ml. In addition, the constancy of the utilisation of glucose, in the presence of insulin, during the period of declining concentration of glucose, would be a fortuitous observation, although it was repeatable at all concentrations of glucose, if the figure of 6.0 ml. were grossly in error. It is improbable that the variation of the volume of recirculated perfusate from the assumed value was greater than 0.5 ml. or, approximately, 8.5%, between experiments.

The accuracy of the determination of the rate of change of the concentration of perfusate is difficult to assess. However the close agreement between the experimental and the predicted concentrations of glucose which is shown in Table 14 suggests that a fourth order polynomial accurately describes the time-course of the concentration. This close agreement

TABLE 15

THE CONTRIBUTION OF TERMS WHICH INCLUDE THE RATE OF CHANGE OF GLUCOSE CONCENTRATION OR THE DIFFERENCE IN CONCENTRATION BETWEEN INFUSATE AND PERFUSATE GLUCOSE TO ESTIMATES OF GLUCOSE UTILISATION

Utilisation = $1/w[\,i(a - x) - P \cdot dx/dt\,]$, where w is the true solid weight of the heart and the remaining symbols have the significance ascribed in the text.

I. A heart perfused without insulin

w = 0.1394 g.; a = 0.494 mg./ml; i = 30.63 ml./hour; P = 6.0 ml.

Time min.	x mg./ml.	dx/dt mg./ml./min. x 10^3	$1/w\,[i(a - x)]$ mg./g. true solid/hour	$-1/w \cdot P \cdot dx/dt \cdot 60$ mg./g. true solid/hour	Utilisation mg./g. true solid/hour
0	0.414	--	--	--	--
10	0.372	-2.150	26.90	+5.55	32.45
20	0.365	0.462	28.46	-1.19	27.26
30	0.376	1.638	25.91	-4.23	21.68
40	0.394	1.788	22.00	-4.62	17.38
50	0.410	1.324	18.50	-3.42	15.08
60	0.420	0.658	16.30	-1.70	14.60
70	0.424	0.202	15.47	-0.52	14.95
80	0.426	0.366	14.99	-0.95	14.04
90	0.434	--	--	--	--

TABLE 15 (cont.)

II. A heart perfused with insulin (100 mU./ml.)

w = 0.1393 g.; a = 0.253 mg./ml.; i = 25.965 ml./hour; P = 6.0 ml.

Time min.	x mg./ml.	dx/dt mg./ml./min. x 10^3	$1/w\,[i(a - x)]$ mg./g. true solid/hour	$-1/w \cdot P \cdot dx/dt \cdot 60$ mg./g. true solid/hour	Utilisation mg./g. true solid/hour
0	0.234	--	--	--	--
10	0.163	-5.143	16.83	+13.29	30.12
20	0.126	-2.396	23.66	+ 6.19	29.85
30	0.111	-0.784	26.46	+ 2.02	28.48
40	0.108	-0.028	27.11	+ 0.07	27.18
50	0.109	0.150	26.93	- 0.39	26.54
60	0.110	0.026	26.74	- 0.07	26.67
70	0.109	-0.120	26.84	+ 0.31	27.16
80	0.108	-0.012	27.03	- 0.03	27.06
90	0.111	--	--	--	--

indicates that the use of the predicted values in making estimates of glucose utilisation is justifiable. Further, the predicted values are necessarily related by the continuous changes in concentration which would be expected to occur in the perfusate. The good fit of the regression lines can also be taken to indicate that the estimates of the rate of change of concentration are reliable, although the order of their accuracy cannot be specified. The error in the product of the volume of the recirculated perfusate and the estimate of the rate of change of concentration is therefore also uncertain. However it is probable that this measure of the rate of change of the amount of glucose in the perfusate is only untrustworthy at times 0 and 90 minutes when the estimates of the slope of the regression line are anomalous.

Clearly, the smaller is the contribution of the rate of change of the amount of perfusate glucose to the estimate, the greater the certainty with which the utilisation of glucose can be estimated at intervals throughout a perfusion. This term could contribute as much as 50% to the estimate which is made after 10 minutes of perfusion. At all subsequent times, whether the hearts were perfused with or without insulin, at least 75% of the estimate of utilisation was due to the difference between the rates of addition and removal of glucose by the infusion and withdrawal of perfusate. The relative contributions of the two terms to the estimation of the time-course of utilisation are shown for two experiments in Table 15. In one of these insulin was included in the perfusate.

TABLE 16

PROPERTIES OF METABOLICALLY UNSTABLE HEARTS

Two examples are given of hearts which were perfused without added insulin and did not attain a steady state of metabolism.

	Time (min.)	Glucose concentration mg./100 ml.	Rate of change of concentration mg./100 ml./10 min.	Glucose utilisation mg./g. true solid/hour	Lactate concentration mg./100 ml.
I	0	10.43	--	--	--
	10	8.92	-1.08	10.41	0.6
	20	8.17	-0.47	10.39	--
	30	7.89	-0.14	10.14	< 0.5
	40	7.82	-0.03	10.00	--
	50	7.77	-0.08	10.23	0.7
	60	7.61	-0.25	10.97	--
	70	7.25	-0.48	12.25	1.1
	80	6.65	-0.71	14.00	--
	90	5.84	--	--	0.8
II	0	8.25	--	--	--
	10	6.37	-1.13	10.65	1.6
	20	5.77	-0.18	9.46	--
	30	5.85	0.26	8.19	1.0
	40	6.16	0.32	7.40	--
	50	6.40	0.14	7.36	< 0.5
	60	6.40	-0.14	8.06	--
	70	6.13	-0.37	9.20	0.6
	80	5.72	-0.41	10.17	--
	90	5.41	--	--	< 0.5

The Time-Course of Utilisation in a Failing Heart

When the object of an experiment is to analyse the content of glucose in a heart which has been perfused at a constant concentration of glucose, stability of the properties of the heart in the final minutes of the perfusion is essential. Evidence has been presented to show that this stability can be observed in hearts which were perfused for 90 minutes. However, when an estimate of glucose utilisation at a constant concentration of perfusate glucose is the sole object of an experiment, the determination of the time-course of utilisation has the advantage that this estimate can be made despite a subsequent failing of the heart. Loss of stability in the properties of hearts which were perfused without exogenous insulin, and at low concentrations of perfusate glucose was found in approximately one-third of such experiments, although no visible deterioration in the condition of the preparations was noted. Under all other experimental conditions, instability was found in less than 25% of the experiments.

In Table 16, two examples are shown of hearts which failed comparatively early in 90 minutes of perfusion. The examples have been selected from those hearts which were perfused in the absence of insulin, and at a low concentration of glucose, to include one experiment in which the rate of utilisation fell initially and one in which the rate remained constant during the early stages of the perfusion. In the latter experiment (Table 16, I), the stability of the preparation in the first 50 minutes of perfusion is evident and the subsequent deterioration is undoubted. The levels of lactate in the perfusate, which were determined at 20 minute intervals, rose after an initial decline. Preparations which remained stable throughout an experiment showed no

increase in the rate of lactate formation from the level which was established after 30 minutes of perfusion. The initial higher level of lactate was attributed to the loss from the heart of lactate which was formed under hypoxic conditions prior to perfusion. Because the rate of glucose utilisation was initially constant, the concentration of perfusate glucose would be expected to attain a constant level after roughly 50 minutes of perfusion. In this experiment, the fall in the concentration of glucose between 40 and 50 minutes was insignificant and the concentrations at these times were taken to be indicative of a constant level. Thus the utilisation and concentration of glucose at 40 and 50 minutes were accepted as being valid estimates made under stable conditions.

When the rate of utilisation fell initially, as is the case in the second example in Table 16, there was greater uncertainty in the interpretation of the results. The rates of utilisation, which were observed at 40 and 50 minutes of perfusion, can be supposed to be those of a stable preparation, because their constancy conforms with the characteristics of hearts whose uptake of glucose was constant in the final 40 minutes of perfusion after declining in the initial 40 to 50 minutes. The fall in the concentration of lactate during the initial period of perfusion is also typical of hearts which attained stability after this period. However, the best estimate of the constant concentration of perfusate glucose is given by the values which were observed at 50 and 60 minutes of perfusion. The identity of these values is of dubious significance because of the opposite direction of the change in concentration at these times and because of the delay, which must be expected, in establishing a constant concentration of

glucose after the attainment of a constant rate of utilisation. If it is assumed that the estimates of utilisation at 40 and 50 minutes are representative of a stable preparation, it can be calculated that the constant concentration of glucose, which would be established at this rate of utilisation and at the rate of infusion of glucose in this experiment, is 6.54 mg./100 ml. The values of the concentration of perfusate glucose at 50 and 60 minutes might therefore underestimate the constant concentration of perfusate glucose by 2.2%.

The examples which have been discussed are extreme in that the failure of these hearts was sufficiently late for useful information to be extracted from the experiments only by assuming that the early behaviour of the hearts conformed to that observed in preparations whose stability was maintained in the final stages of their perfusion. When the deterioration of the condition of a preparation occurred later than was the case in the two examples, the interpretation of the results required no assumptions. Earlier failure than in the examples was rare, and the results which were obtained in such circumstances were disregarded.

CHAPTER FIVE

DISCUSSION

In principle, the apparatus for cardiac perfusion which was used in this work enables a heart to be perfused at a constant concentration of glucose and also enables the utilisation of glucose, at that concentration, to be measured. For the purposes of this investigation, a steady state of permeation of glucose has been assumed to exist when a constant concentration of glucose is maintained in the perfusate of a heart.

The results of the experiments which have been presented in this Section show that, within the limits of the accuracy of the methods for the estimation of glucose, a constant concentration of perfusate glucose could be established and maintained in the perfusate of a heart, whether or not insulin was included in the perfusate. In this circumstance, the rate of glucose utilisation was necessarily constant since the rate of addition of glucose to the perfusate was also constant. To this extent, a steady state was established in the preparations.

The time-course of glucose utilisation in the approach to a steady state reveals strikingly the initial rapid change in the metabolic properties of hearts, which are perfused in the absence of exogenous insulin and at a high concentration of perfusate glucose. A decline in the permeability of the perfused rat heart to non-metabolised sugars was observed, by Zachariah

(1961) and Gilbert (1963), to occur in the first 30 to 40 minutes of perfusion. In this work, the decline in glucose utilisation was more prolonged, constancy being reached after 40 to 50 minutes of perfusion. It was not possible to confirm that this decline in utilisation was associated with the loss of endogenous insulin from the preparation. However, Zachariah (1961) found that the injection of anti-insulin serum into rats one hour before removing their hearts reduced the initial permeability of the isolated hearts to a non-metabolised sugar.

The failure to observe an effect of anti-insulin serum on the utilisation of glucose when hearts were exposed to the serum in vitro has several possible explanations. First, it is possible that anti-insulin serum, which is prepared from the blood of guinea-pigs which have been immunised by repeated injections of ox and pig insulin, may be inactive towards the insulin of rats. Secondly, if the serum were active, it is possible that the antibody protein might not be able to pass through the capillary walls, in which case it could only neutralise that insulin which is released from the membranes or the interior of the myocardial cells and enters the perfusate. On the supposition that insulin which enters the perfusate in the absence of the serum is adsorbed and inactivated on the surfaces of the perfusion apparatus, the inclusion of anti-insulin serum in the perfusate would not affect the rate of the elimination of insulin from the system. Thirdly anti-insulin serum may not be able to bind insulin which is itself bound to, say, the membrane. No attempt was made to examine the properties of hearts from animals which had been treated with anti-insulin serum, because, whatever might be the case for a non-metabolised sugar, the results would certainly

be inconclusive if the utilisation of glucose by such hearts were studied. Elimination of endogenous insulin remains the most probable explanation of the decline in the uptake of glucose by hearts which are perfused in the absence of added insulin and at relatively high concentrations of perfusate glucose.

The comparative constancy of the utilisation at low concentrations of glucose in the absence of insulin contradicts this interpretation because the content of endogenous insulin of all the preparations which were used in this work must be similar. A constant low rate of glucose utilisation suggests that at low concentrations of perfusate glucose, the endogenous insulin is either ineffective in promoting utilisation or is eliminated rapidly from the preparation under these conditions. If insulin is assumed to complex with a carrier in the membranes of cardiac cells, the latter explanation requires that insulin is bound less firmly at low concentrations of perfusate glucose. This circumstance could be envisaged if the affinity of insulin for a complex of the carrier and glucose is greater than its affinity for the free carrier. While this explanation is feasible, the alternative, that insulin is ineffective in promoting glucose utilisation at low concentrations of glucose, has the merit that more evidence can be adduced in its support. It will be shown, p.176, that the parameters for glucose permeation for the free carrier and the insulin-carrier complex, which have been estimated in this work, are consistent with the supposition that, when the two carriers are present together, the insulin-carrier complex contributes relatively little to the transfer of glucose at low concentrations of perfusate glucose.

In this connection, it may be noted that the results of Morgan,

Henderson et al. (1961) are in accord with the observations which have been presented. These workers estimated the utilisation of glucose by the perfused rat heart from the fall in the concentration of a known volume of recirculated perfusate in the first 30 minutes of perfusion. In their experiments, in which insulin was not added to the perfusate, endogenous insulin would be expected to influence the rate of utilisation. When the concentration of perfusate glucose was greater than 50 mg./100 ml., the utilisation approximated to a rate which, in this work, was observed only in the presence of insulin. At a low concentration of glucose, the rate of utilisation was similar to that which was found, in this work, at the same concentration in the absence of the hormone.

The presumed influence of endogenous insulin on the metabolic properties of the preparations was so prolonged that a pre-perfusion for at least 30 minutes, and preferably for 50 minutes, is necessary before it can be reasonably claimed that a heart which is taken from a fed animal is in an insulin-free state. In this investigation, the further perfusion which is necessary to establish a constant concentration of perfusate glucose resulted in the adoption of 90 minutes as the standard duration of an experiment. Beyond this time, the condition of those hearts which were perfused without insulin inevitably deteriorated. Although a higher rate of infusion than 0.5 ml./min. would have allowed a more rapid attainment of a steady state, it would also have resulted in a reduced accuracy in the estimation of utilisation. The experimental conditions of this work enabled the utilisation of glucose to be measured accurately except at concentrations of perfusate glucose greater than 100 mg./100 ml. in the absence of insulin

and provided a compromise between reduced accuracy of estimation with a shorter time of perfusion and the deterioration of the condition of the preparation with longer perfusion.

CHAPTER SIX

SUMMARY

1. Precise methods for the estimation of glucose are essential for the determination of the difference between the concentrations of infusate and perfusate glucose on which the calculation of the rate of glucose utilisation by a perfused heart depends. Automated methods of glucose estimation have been described and have been shown to permit the accurate measurement of utilisation.

2. The time-course of the concentration of perfusate glucose has been followed when hearts were perfused with and without exogenous insulin. Treatment of the isolated heart with anti-insulin serum did not alter the pattern of the time-course which was observed in the absence of insulin.

3. Fitting a fourth order polynomial to the time-course of the concentration of perfusate glucose allowed the determination of the rate of change of concentration at any time in the perfusion. This information contributed to the calculation of the time-course of utilisation. Except at low concentrations, the utilisation of glucose, in the absence of exogenous insulin, declined in the first 40 to 50 minutes of perfusion to a constant rate. Despite the lack of an effect of anti-insulin serum, this decline has been attributed to the elimination of endogenous insulin from

the hearts. In the presence of insulin, a constant rate of utilisation was maintained throughout the period of perfusion.

4. Perfusion for 90 minutes established a constant concentration of glucose in the perfusate of the hearts, which, for the purposes of this investigation, were assumed to be then in a steady state.

SECTION III

THE MEASUREMENT OF INTRACELLULAR GLUCOSE

CHAPTER ONE

I N T R O D U C T I O N

The conclusion was reached, in the preceding section, that a steady state of glucose utilisation and, therefore, of permeation was established in a metabolically stable preparation by the technique for cardiac perfusion which was developed for this investigation. In such a preparation, the utilisation of glucose can be measured at a well defined concentration of perfusate glucose. With the additional knowledge of the concentration of intracellular glucose, the parameters of the process of permeation might be determined. In addition, the kinetics of the phosphorylation of glucose and the effect of insulin thereon might also be studied.

The difficulties of the unexceptionable estimation of the concentration of the intracellular glucose of a perfused heart are as evident as the advantages which the estimation would yield. Inhibition of glucose metabolism must be achieved so rapidly after the cessation of perfusion that the amount of glucose in the organ remains that of the steady state. The extraction of the glucose from the heart must be complete and the determination of the amount must be precise. The distribution of glucose between the extracellular and intracellular fluids has then to be established, and in doing so the influence of the compartmentation of these fluids on the local concentrations of the sugar must be taken into account. Knowledge of the volumes of extra-

cellular and intracellular water is required. The determination of the contribution of the extracellular and the intracellular glucose to the total glucose of a heart will be discussed in some detail in this chapter, not only for its relevance to this Section, but also because inherent in this problem are considerations fundamental to the interpretation of the kinetics of glucose utilisation which is adopted in the following Section.

Determination of the Volume of the Extracellular Water

The volume of extracellular water in a perfused organ may be measured by the inclusion in the perfusate of a substance which does not penetrate the cells of the tissues but which diffuses readily from the capillaries. A uniform concentration of this "marker" should be established throughout the extracellular water and should be equal to the concentration of the marker in the perfusate. If the amount of the marker in the organ can be measured, the "space" of the substance may be calculated. The term "space" will be used to mean the volume through which a known amount of a substance would be distributed in a heart at the concentration of the substance in the perfusate. In the present context, the space of an extracellular marker is equated with the volume of extracellular water.

This method for the determination of the volume of extracellular water is justified by the closely comparable results which have been obtained when substances of very different molecular dimensions, for example, sorbitol, raffinose and inulin, were used as markers. Fisher and Young (1961) also demonstrated the equality of the volume of liquid which is expressed from a rat heart when subjected to a controlled pressure with the space occupied

by an extracellular marker.

Inulin and raffinose were used in this investigation for the measurement of the volume of extracellular water. Their estimation is described in the third chapter of this Section.

The Determination of the Amount of Extracellular Glucose

If it may be assumed that the concentration of glucose throughout the extracellular water is the same as that in the perfusate, the amount of the total cardiac glucose which is extracellular can be determined when the extracellular volume and the concentration of perfusate glucose are known. This assumption requires that the glucose space in a heart must be at least as great as the space which is occupied by an extracellular marker. Their inability to satisfy this requirement, when hearts were perfused without insulin, led Morgan, Henderson et al.(1961) and Neely et al.(1967$_b$) to question the assumption and to suggest that a significant difference must exist between the concentrations of glucose in the intracapillary water and in the interstitial water. Clearly, it is inevitable that the concentration of a metabolisable substance at the outer surface of the membrane of myocardial cells be less than the concentration in the intracapillary water. The magnitude of this difference in concentration, for glucose, cannot be properly defined by measuring the deficiency between the observed amount of glucose in a heart and a theoretical amount, which is based on the original assumption, without any certainty that the arrest of metabolism is instantaneous, the extraction of glucose from a heart is complete or that the amount of intracellular glucose is negligible. It is therefore necessary to

attempt to assess by other means the probable magnitude of the difference between the concentrations of glucose in the compartments of the extracellular water.

The passage of a solute from the intracapillary water into and through the interstitial water is affected by numerous factors, of which the influence of the bulk flow of liquid across the capillaries of a contracting muscle should not be neglected (Stubbs and Widdas, 1959; Young, 1968). The capillary permeability coefficient for the solute is of fundamental importance in determining the rate of permeation, but its application, and often its derivation, depends upon a reliable estimate of the surface area of a capillary network. Even when such an estimate is available, the uncertainty of the effect of the continuous variation in the capillary patency under nervous, hormonal and mechanical influences makes the distinction between the potential and the useful surface area important but difficult to quantify. The capillary permeability coefficient and the capillary surface area may not determine the maximum rate of permeation of some solutes; for a rapidly permeating substance, the rate of perfusion of a tissue may be limiting (Schafer and Johnson, 1964). Although these factors should largely determine the magnitude of the concentration difference across the capillary wall which is established at any rate of glucose utilisation, gradients may also be supposed to exist in both the capillary water and the interstitial water. In a tissue which is perfused by an artificial perfusate the intracapillary concentration of a metabolisable solute may show radial variations, whereas the passage of blood through a capillary bed may be treated as block flow with little or no radial variations in concentration because of the displacement of boundary layers by the

erythrocytes. However the repeated occlusion of the capillaries of a contracting muscle would presumably disrupt laminar flow. Within the interstitial water, the magnitude of a concentration gradient would be determined by the rate of utilisation, the diffusion coefficient of the substance, the distance between the capillary and the myocardial cell, and by the extent to which the contractions of the muscle fibres exert a stirring effect in the compartment.

Schafer and Johnson (1964) have calculated the radial diffusion half-time for the diffusion of sucrose and inulin through the interstitial water of the rabbit heart. For each solute, the calculated half-time was far less than the observed half-time for the approach to the constant volume of distribution of these extracellular markers. Diffusion through the interstitial water was not therefore the rate-limiting step. This conclusion is likely to be also valid for the passage of glucose through the extracellular water to the myocardial cells of the rat because the calculated half-times for radial diffusion depend only on the spatial relationships of the capillaries and muscle fibres, and the diffusion coefficients of the solutes in the interstitial water. There is little variation between species in the density of capillaries in the myocardium (Shipley, Shipley and Wearn, 1937; Gallo, 1956; Gregg and Fisher, 1963) and large differences between species in the diffusion coefficients of solutes in interstitial water is improbable. There is little doubt that any lack of uniformity in the concentration of extracellular glucose must lie in a difference between the concentrations of the sugar in the vascular and interstitial compartments, and must be created by restrictions imposed by the capillary walls on the passage of glucose between the

compartments.

The assumption that the histology of the myocardium is similar in different species appears to be justifiable but it does not necessarily follow that the permeability coefficients of the cardiac capillaries in different species may be assumed to be the same. There is also a possibility that the structural integrity of the capillaries in an isolated perfused heart may vary with the species from which the heart is taken. The work of Sutherland and Young (1966) on the distribution of an Evans blue-albumin complex in the rat heart, in vivo and when perfused in isolation, indicated a considerable increase in the permeability of the capillaries of the isolated heart. In vivo, the complex did not penetrate the interstitial water, but within two minutes of perfusion of the isolated heart, extensive penetration had occurred. The authors concluded that the change in the permeability of the capillaries took place as a consequence of the excision of the heart, because the extent of the penetration of the interstitial water did not depend on the period of perfusion before which the heart was exposed to the complex. A varied response of animals from different species to the shock of anaesthetisation and the excision of an organ appears probable so that the permeability coefficient of the cardiac capillaries in one species would be inapplicable to another. It has therefore been assumed, on the evidence of the work of Sutherland and Young (1966), that the loss of integrity in the structure of the capillaries of the isolated rat heart largely removes any restriction which the capillary wall might impose on the passage of glucose into the interstitial water. The concentration of glucose has been treated as uniform throughout the extracellular water. If, however,

it is supposed that the permeability coefficients of the cardiac capillaries of the isolated rabbit heart are applicable to the rat heart, the values found by Schafer and Johnson (1964) for sucrose and inulin can be used to give an estimate of the coefficient for glucose.

Schafer and Johnson (1964) estimated the permeability coefficient of rabbit capillaries to sucrose to be in the range 5×10^{-5} to 20×10^{-5} cm./sec. It is reasonable to suppose that the permeability coefficient to a compound of smaller molecular size, such as glucose is greater than that to sucrose. If the capillary density in myocardial tissue is taken as 3.5×10^{5} capillaries per sq. cm. (Schafer & Johnson, 1964; Gregg & Fisher, 1963) and the radius of a capillary as 5×10^{-4} cm., the capillary surface area is approximately 1100 sq. cm./cc. of tissue. The ventricular muscle of the rat hearts which were used in this work had a volume of about 0.8 cc. Taking the surface area of the capillaries of the rat heart to be 900 sq. cm. and the permeability coefficient to glucose to be 20×10^{-5} cm./sec., the concentration difference across the capillary wall varies from 0.5 to 1.5 mg./100ml. as the rate of glucose utilisation varies from 18 to 54 mg./g. dry weight/hour. Since hearts perfused with insulin were found to use about 20 mg./g. dry weight/hour at a concentration of perfusate glucose of 5 mg./100 ml., the concentration of interstitial glucose might have been overestimated by 10%. It is, however, impossible to assess the magnitude of the errors inherent in this calculation.

The Concentration of Intracellular Glucose

The amount of intracellular glucose is given by the difference between

the total glucose in a heart and the amount of glucose which is extracellular, so that, when this difference is small, a small percentage error in the determination of the extracellular glucose may lead to a large percentage error in the estimate of intracellular glucose. The concentration of intracellular glucose depends upon the volume of the intracellular water which is accessible to glucose.

The volume of intracellular water is determined by the difference between the volume of extracellular water and the total water of the heart, which, in turn, is given by the difference between the weight of the organ when wet and when dry. Morgan, Henderson et al.(1961) assumed that only 75% of the intracellular water was accessible to glucose. This assumption was based on studies of the distribution of non-metabolised sugars. Details of these studies were not given, but it may be presumed that the permeation of the perfused heart by a non-metabolised sugar reached equilibrium when the concentration of intracellular sugar, calculated from 100% accessibility of intracellular water, was still less than the extracellular concentration. It might then be supposed that the sugar was distributed through an intracellular volume such that the intracellular and extracellular concentrations were equal. However, the carrier hypothesis predicts that, when the extracellular concentration of a sugar is much greater than the half-saturation constant of the carrier for the sugar, the approach to equilibrium will become infinitely slow although the intracellular concentration of the sugar is still much less than the extracellular concentration.

Gilbert (1963) found that, at a sufficiently low extracellular concentration of a non-metabolised sugar, the intracellular concentration

could become equal to the extracellular concentration when he took the accessibility of the intracellular water to pentose to be unrestricted. Any lower degree of accessibility would require the postulation of the transport of the sugar against a concentration gradient, for which there is no evidence. In this work, the intracellular water has been assumed to be fully accessible to glucose which is at a uniform concentration.

A Criterion for the Complete Inhibition of Metabolism

The basis for the assumption that the extracellular concentration of glucose, in a perfused heart, is uniform and equal to the concentration of perfusate glucose has been discussed. If the assumption is tenable, successful techniques for the inhibition, at the cessation of perfusion, of the metabolism of glucose and for the subsequent extraction of the glucose from the heart, will result in an estimate of the glucose space which is invariably greater than the volume of extracellular water.

Attempts to satisfy this criterion and to achieve the unexceptionable determination of the amount of glucose in extracts of cardiac muscle are the subjects of the following chapters.

CHAPTER TWO

THE ESTIMATION OF GLUCOSE IN EXTRACTS OF CARDIAC MUSCLE

INTRODUCTION and METHODS

An accurate estimate of the amount of glucose in a heart is a prerequisite for the determination of the concentration of the intracellular glucose. No satisfactory method for achieving this end was found.

The experiments about to be described were concerned with the measurement of the apparent concentration of glucose in extracts of rat cardiac muscle to which known amounts of glucose had been added. Extracts were made from hearts which had been washed free of blood on the apparatus shown in Fig. 13 or which had, in addition, been perfused for one hour without an exogenous source of nutrient, so that the amount of glycogen in the hearts would be reduced. The hearts were then subjected to a procedure for attaining the arrest of metabolism. A detailed description of the procedure and the manner in which it was assessed are given in the following chapter. In essence, the heart was frozen by clamping it between two blocks of aluminium, which had been cooled in liquid nitrogen, and, after initial fragmentation with bone forceps, it was further disrupted by ultrasonication in a vessel which contained ethanol and was surrounded by a mixture of acetone and solid carbon dioxide. Removal of the ethanol in a freeze-drying

TABLE 17

THE RECOVERY OF GLUCOSE FROM EXTRACTS OF UNPERFUSED HEARTS

Hearts were washed free of blood and extracts (25 ml.) were made with water at 100° C. or room temperature. Occasionally, the tissues of two hearts were pooled for extraction (50 ml.). Known amounts of glucose were added to 10 ml. aliquots of the extracts, and the apparent amount was estimated after deproteinisation.

1. Extracts made at 100° C.

Added glucose (µg.)	Difference between the estimated and the added amount of glucose (µg.)						
100	+ 8.0	+21.6	+ 7.0	+21.4	+12.3	+21.7	--
150	-14.0	+18.4	+12.2	-24.5	+14.7	+30.3	--
200	+40.1	+18.7	+ 6.4	- 6.6	--	--	--
250	+28.8	+17.7	+ 5.3	- 6.6	--	--	+19.3
300	--	--	--	--	--	--	+19.6
No. of hearts	2	2	2	2	1	1	1

II. Extracts made at room temperature

Added glucose (µg.)	Difference between the estimated and the added amount of glucose (µg.)					
100	+10.3	--	--	--	--	--
150	+13.6	--	--	--	--	--
200	+16.6	-11.0	+29.3	+16.0	-33.2	+39.4
250	+13.2	--	--	--	--	--
300	--	- 7.5	+15.8	+75.0	-50.7	-38.0
No. of hearts	2	1	1	1	1	1

apparatus yielded a powder and, commonly, one or two small pieces of what appeared to be largely connective tissue.

Extracts were made from the dry cardiac tissue by its repeated suspension in 5 ml. of water for 15 minutes. The supernatants which were obtained after centrifugation in five repetitions of this procedure were pooled and made up to a final volume of 25 ml. Occasionally, the fragments of two frozen hearts were disrupted together by ultrasonication. The dried tissues were then extracted five times with 10 ml. of water to give a final volume of 50 ml. The extractions were made either at room temperature or by placing the suspension in a boiling water bath. Known amounts of glucose (100 to 300 µg.) were added to 10 ml. aliquots of the extracts. The whole was deproteinised by the precipitation of zinc hydroxide (procedure described on p. 75) to give a final volume of 25 ml.

RESULTS

The Return of Glucose from Extracts of Unperfused Hearts

The differences between the estimated amount and the added amount of glucose in 10 ml. of extracts from hearts which were not perfused other than for the elution of blood are shown in Table 17. The results do not reveal any common characteristic. Although in some cases the differences between the observed and the added amounts of glucose were similar for samples from the same extract, this was not always found, and, indeed, the return of glucose could be both greater and less than 100%. The temperature at which the extracts were made did not affect the quality of the

TABLE 18

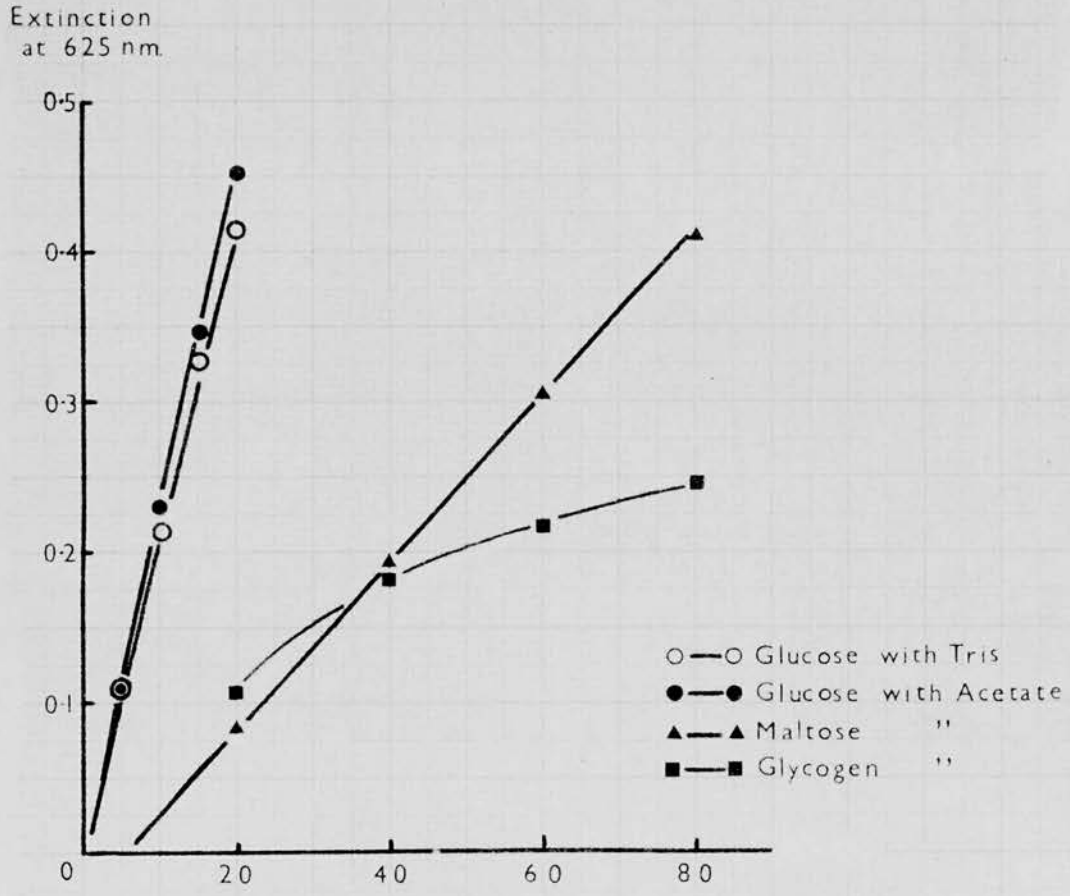
THE RECOVERY OF GLUCOSE FROM EXTRACTS OF PERFUSED HEARTS

Hearts were perfused without exogenous nutrient for one hour and were extracted (to 25 ml.) with water at room temperature. Known amounts of glucose were added to 10 ml. aliquots of the extracts and the apparent amount of glucose estimated after deproteinisation.

Added glucose (µg.)	Difference between the estimated and the added amount of glucose (µg.)				
200	- 4.0	+16.7	- 1.2	- 7.3	--
300	+44.0	+16.9	-22.4	+37.2	- 7.9
No. of hearts	1	1	1	1	1

Figure 30.

The Effect on the Specificity of a Reagent, for the Estimation of Glucose, of Preparation in a "Tris" Buffer (pH 7.0)



Standard solutions of glucose, maltose and glycogen reacted in the AutoAnalyser with the reagent for glucose estimation, with gum guaiacum resin as chromogen, which was prepared in a Tris/HCl buffer (0.4M, pH 7.0) or a Sodium Acetate/Acetic acid buffer (0.4M, pH 5.5). No reaction of maltose or glycogen was detected with the former reagent.

results. Deproteinisation of extracts without the addition of glucose yielded solutions which gave detectable colour in the Autoanalyser. The reaction was too slight to be quantified.

The Return of Glucose from Extracts of Perfused Hearts

The differences between the estimated amount and the added amount of glucose in 10 ml. of extracts made at room temperature from hearts which were perfused for an hour without an exogenous source of nutrient, are shown in Table 18. The variability of the results, which were obtained with samples of the same extract, was again found. Although there is a preponderance of estimates in which the return of glucose was less than 100%, there is no difference of any statistical significance in comparison with those results in Table 17, which were obtained with extracts made at room temperature.

The Analysis of Glucose in Extracts of Cardiac Muscle with a Modified Reagent

Impure preparations of glucose oxidase may be contaminated by disaccharidases (Dahlqvist, 1961) and by sufficient amylase for a preparation to be used in a method for the estimation of glycogen (Rerup and Lundquist, 1967). Glycogen in concentrations of 20 to 80 µg./ml. gave considerable colour in the automated system for the estimation of glucose, in which gum guaiacum resin was the chromogen. Maltose also reacted in the same system (Fig. 30). The use of Fermcozyme as a source of glucose oxidase does not, therefore, permit the specific estimation of glucose when the reagent is

prepared in an acetate buffer and the sample of glucose also contains maltose and glycogen.

Maltase is considerably reduced in activity in a Tris buffer, pH 7.0, (2-Amino-2-(hydroxymethyl)propane-1:3-diol and hydrochloric acid) in comparison with its activity in a phosphate buffer pH 7.0, whereas the activity of glucose oxidase is similar in the two buffers (Dahlqvist, 1961). This observation suggested that a more specific method for the estimation of glucose might be possible with the substitution of a Tris buffer, pH 7.0 (1.0 M) for the acetate buffer, pH 5.6, in the preparation of a solution of gum guaiacum resin by the procedure which was described on p. 79. The two reagents will be termed "Tris" and "Acetate".

Properties of the "Tris" Reagent

The "Tris" reagent was used with the Autoanalyser in the system which was described for the "Acetate" reagent p. 73.

a) Reaction with Glycogen and Maltose

No reaction which produced a compound absorbing at 625 nm was detected between the "Tris" reagent and solutions of glycogen at 20 to 80 µg./ml. With maltose, reaction was either undetectable or weak - a variation which possibly reflects differences in the batch of gum guaiacum used in the preparation of the reagents. However although reaction with maltose was occasionally detectable, the extinction was less than 0.01 for a maltose solution of 80 µg./ml.

b) Reaction with Glucose

The relationship between extinction and the concentration of glucose in samples which were estimated with the "Tris" reagent are also shown in

TABLE 19

EFFECT OF DEPROTEINISATION WITH ZINC HYDROXIDE ON GLUCOSE ESTIMATION WITH GUM GUAIACUM AS CHROMOGEN IN "TRIS" BUFFER

Zinc hydroxide was precipitated (from 2 ml. 5% $ZnSO_4$) in four solutions containing known amounts of glucose. The apparent amounts of glucose in the filtrates of these solutions were determined.

Added glucose (µg.)		150.0		200.0
Estimated glucose (µg.)	a)	149.1	a)	197.2
	b)	150.4	b)	200.0
Mean estimate		150.2		198.6
Percent. recovery		100.1		99.3

TABLE 20

EFFECT OF THE METHOD OF ESTIMATION ON THE RECOVERY OF GLUCOSE FROM EXTRACTS OF CARDIAC MUSCLE.

Aqueous extracts (25 ml.) were made at room temperature of hearts which were either washed free of blood or perfused without exogenous nutrient for one hour. Known amounts of glucose were added to 10 ml. aliquots of the extracts. The apparent amount of glucose was estimated in the one sample with two reagents, both containing glucose oxidase, peroxidase and gum guaiacum but one prepared in acetate buffer (0.4 M, pH 5.5) and the other in Tris buffer (0.4 M, pH 7.0).

I. Hearts washed free of blood

Added glucose (µg.)	Reagent	Percent. recovery from hearts			
		1	2	3	4
200	"Acetate"	94.5	114.6	108.0	83.4
200	"Tris"	98.6	97.8	83.4	34.9
300	"Acetate"	97.5	105.8	125.0	83.1
300	"Tris"	70.2	83.8	103.2	33.3

II. Hearts perfused without nutrient

Added glucose (µg.)	Reagent	Percent. recovery from hearts			
		1	2	3	4
200	"Acetate"	98.0	108.3	99.4	96.3
200	"Tris"	56.9	99.0	87.9	50.0
300	"Acetate"	85.3	105.6	92.5	112.4
300	"Tris"	45.2	99.2	69.5	68.4

Fig. 30. As with the "Acetate" reagent (Fig. 23), there is a departure from a linear relationship at the highest concentration of glucose. Below 15 µg./ml., there was no significant departure from linearity. The sensitivity of the estimation of glucose was similar with the two reagents.

c) The Effect of Deproteinisation

No effect of the precipitation of zinc hydroxide in solutions of glucose of known concentration was detected in the subsequent analysis of the solutions. The results of such an experiment are shown in Table 19.

The Recovery of Glucose from Extracts of Cardiac Muscle

The properties of the "Tris" reagent show that this reagent provides a more specific method for the estimation of glucose than does the "Acetate" reagent. The recovery of glucose from extracts of hearts which had been perfused without glucose or were unperfused save for the elution of blood was determined with both the "Tris" reagent and the "Acetate" reagent. In Table 20, the percentage recoveries are given when the same sample was estimated with both reagents. Estimations made with the "Tris" reagent indicated a significantly lower recovery ($P < 0.001$) than did those estimations which were made with the "Acetate" reagent.

DISCUSSION:

Estimates of the recovery of glucose varied considerably not only between extracts from different hearts but also between samples of the extract of one heart. The latter observation suggests that the source of the variation might have been in the procedure for deproteinisation. No effect of this

procedure was found on the estimation of glucose in solutions which did not contain any extract of cardiac muscle (Tables 6 and 19). In one experiment of the series described in this chapter zinc sulphate and sodium hydroxide were added to a portion of the filtrate obtained after the normal zinc hydroxide treatment. The recovery of glucose was not affected by this treatment. If deproteinisation was a source of error, it therefore appears probable that the effect was in some way associated with the actual removal of protein from solution and not with an influence on the methods for glucose estimation. The results of the estimation of glucose with the "Tris" reagent, after the deproteinisation of an extract of cardiac muscle, indicate that glucose had been lost from solution in the majority of the experiments. It is possible that the adsorption of glucose by protein in the course of deproteinisation might be the cause of the variable and low recoveries of glucose when a comparatively specific method for the estimation of glucose was used. If this interpretation is valid, the high recoveries of glucose, which were measured with the "Acetate" reagent, might be presumed to reflect the presence in the samples of substances which react as glucose with the reagent. The recovery of glucose would therefore be expected to be lower from those extracts which were made from hearts which had a reduced content of glycogen after one hour of perfusion without exogenous nutrient. No significant effect of such perfusions on the recovery of glucose was found. Thus no conclusive explanation can be given of the failure of this investigation to achieve the estimation of glucose in extracts of cardiac muscle under the experimental conditions which were employed.

CHAPTER THREE

THE INHIBITION OF GLUCOSE METABOLISM IN THE PERFUSED HEART

INTRODUCTION

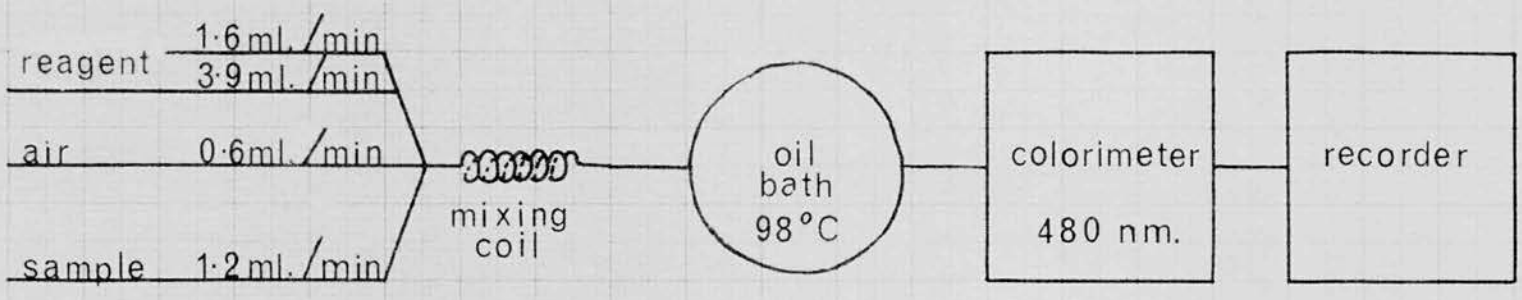
In the introductory chapter to this section, a criterion was proposed for testing the effectiveness of a method for the rapid inhibition of glucose metabolism in a perfused heart. This criterion was based on the assumption that the concentration of extracellular glucose is equal to that of the perfusate glucose, in which case the glucose space should never be less than the volume of extracellular water. Clearly, the proper application of this criterion is dependent upon accurate measurements of both the volume of extracellular water and of the amount of glucose in a heart. The latter measurement was shown in the preceding chapter to be impractical by the methods which were used in that investigation. Associated studies on the inhibition of glucose metabolism could not, therefore, be taken beyond the qualitative level. Nevertheless, the problems inherent in such a study are relevant to the ultimate question of the feasibility of making precise measurements of the concentration of intracellular glucose with the techniques at present available.

Although the total cardiac glucose could not be measured accurately, the criterion for testing a method for the inhibition of glucose metabolism

could be applied when cardiac glucose was estimated with the "Acetate" reagent. With this reagent, the return of glucose from an extract of cardiac muscle was typically but not invariably greater than 100%. If the glucose spaces, which were determined from estimates of total cardiac glucose with the "Acetate" reagent, were consistently less than the volume of extracellular water it could be reasonably concluded that the inhibition of the metabolism of glucose or the extraction of glucose from the tissues was incomplete. On the other hand, glucose spaces in excess of the volume of extracellular water could not be taken as proof of an effective technique.

Initially, an additional test of the inhibition of metabolism at the cessation of perfusion was employed. The amount of lactate in the extract of a heart was determined and compared with the amount of lactate which would be present if the total water of the heart contained the same concentration of lactate as the perfusate. The observed amount of lactate was invariably the larger quantity and the difference between the amounts was taken as a measure of the efficacy of a method for the rapid inhibition of glucose metabolism. However, without knowledge of the real intracellular concentration of lactate, this test allowed only the rejection of an inadequate method and did not prove a method effective. The test was also only applicable when the concentration of perfusate lactate was sufficiently high to be measured accurately and when the amount of lactate in the heart was therefore also measurable. This requirement limited the test to experiments in which hearts were perfused with insulin. There can, however, be no guarantee that a technique which is apparently effective in minimising the

Figure 31.



System for the Automated Estimation of Fructofuranosides

loss of glucose in a heart which contains a relatively large amount of the sugar may not lead to considerable error in an estimate of the concentration of intracellular glucose in a heart which contains little glucose. The relative magnitudes of the glucose space and the volume of extracellular water in a heart provided the better criterion for the assessment of the efficiency of a method for the rapid inhibition of glucose metabolism because the comparison could be made under all experimental conditions.

Determination of the Volume of Extracellular Water

Inulin and raffinose were used as markers of the extracellular water of the heart. Both substances have been shown to be distributed through a fraction of the total water of cardiac and other tissues, which is compatible with their being excluded from the intracellular water (Wilde, 1945; Fisher and Young, 1961; Gilbert, 1963).

Estimation of Inulin and Raffinose

Inulin and raffinose were estimated by an automated method, which was developed by Gilbert (1963) and is applicable to the estimation of all fructofuranosides. The use of the method with the Autoanalyser is illustrated in Fig. 31.

The Composition of the Reagent

1 g. of resorcinol was dissolved in 400 ml. of distilled water. 600 ml. of concentrated hydrochloric acid, in which 0.05 g. of ferric chloride had been dissolved, were added to this solution. The reagent therefore contained 0.1% resorcinol and 0.005% ferric chloride in 60% hydrochloric acid.

Figure 32.

ESTIMATION OF FRUCTO-FURANOSIDES

Compliance with Beer's Law

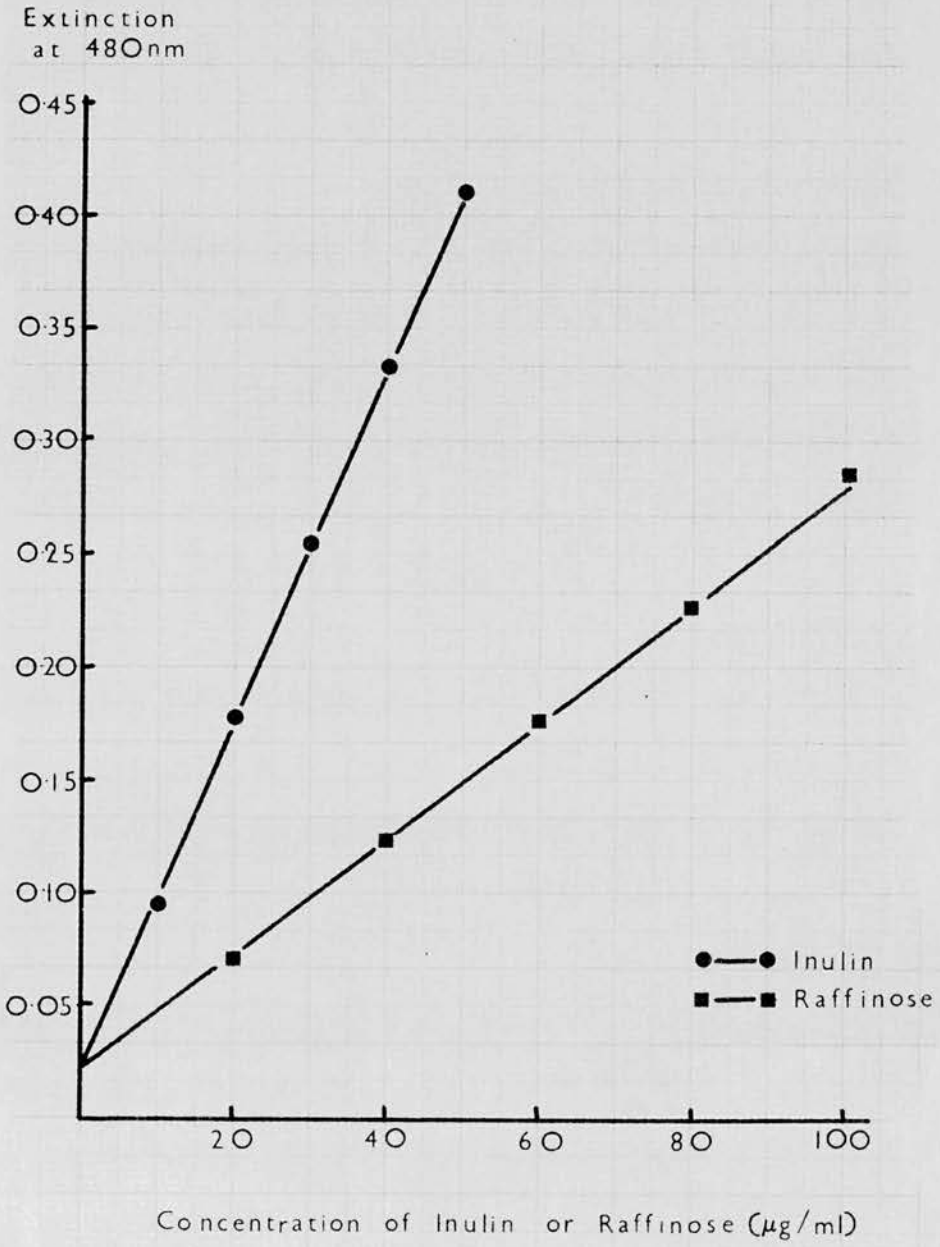


TABLE 21

EFFECT OF GLUCOSE ON THE ESTIMATION OF INULIN

The apparent concentration of inulin was measured in nine solutions containing known amounts of inulin and glucose.

Added inulin (μg./ml.)	Estimated inulin (μg./ml.) in solutions containing		
	2 μg. glucose/ml.	3 μg. glucose/ml.	10 μg. glucose/ml.
10.0	10.0	10.1	10.2
20.0	19.6	19.9	19.9
30.0	29.2	29.2	29.4

TABLE 22

EFFECT OF DEPROTEINISATION WITH ZINC HYDROXIDE ON THE ESTIMATION OF INULIN AND RAFFINOSE

Zinc hydroxide was precipitated (from 2 ml. 5% $ZnSO_4$) in three solutions of inulin and three of raffinose which were all of known concentration. The apparent concentrations in the filtrates were determined.

Added inulin (μg./ml.)	10.0	20.0	30.0
Estimated inulin (μg./ml.)	10.02	20.45	29.96
Percent. recovery	100.2	102.3	99.9
Added raffinose (μg./ml.)	20.0	40.0	60.0
Estimated raffinose (μg./ml.)	20.00	40.44	59.90
Percent. recovery	100.0	101.1	99.8

Compliance with Beer's Law

In Fig. 32 the extinction at 480 nm are plotted against the concentration of inulin or raffinose in sample solutions. Good agreement with Beer's Law was found. There is an approximately three fold difference in the magnitudes of the slopes of the two plots. This difference is in accord with the lower proportion of residues of fructose in the molecule of raffinose.

The Effect of Glucose on the Estimation of Inulin and Raffinose

In Table 21, estimates are given of the concentration of inulin in solutions which also contained glucose at up to 10 µg./ml., which is greater than the concentration of glucose which was present when inulin or raffinose was estimated following a perfusion. At these concentrations, glucose did not interfere with the method.

The Effect of Deproteinisation on the Estimation of Inulin and Raffinose

The precipitation of zinc hydroxide in solutions of inulin and raffinose by the procedure which was described on p. 75, was not, at first, found to lead to anomalous estimates of the concentration of the substances in the filtrates. Results of experiments of this sort are depicted in Table 22. At no time was any evidence found to suggest that this method of deproteinisation might affect the estimation of raffinose. However, this was not so for inulin. In every perfusion a known amount of extracellular marker was included in the perfusate, and its concentration in a deproteinised sample was subsequently determined. In 25 experiments in a period of three months, no anomalous estimates of the concentration of perfusate inulin were obtained, but, thereafter, although no change in procedure was made, the

recovery of inulin from the perfusate became variable and markedly reduced. The experiments depicted in Table 22 were repeated. On occasions, the recovery of inulin was 100%, but this was not consistently found. The recovery was not reproducible when, instead of titrating a solution of zinc sulphate to the end-point of phenolphthalein with sodium hydroxide, the same volume of sodium hydroxide as that determined by titration was added to solutions containing the same concentration of inulin and zinc sulphate. Variations in titration were therefore inadequate to explain the variable recovery of inulin. When sodium hydroxide was added in excess, the percentage recovery fell with increasing pH, but this effect was observed only after the end-point of the titration was exceeded by at least 0.25 ml. of approximately 0.25 N sodium hydroxide. No explanation of the change in the sensitivity of the estimation of inulin to the procedure for deproteinisation was found in these investigations, which were made on inulin from several batches. Consequently, raffinose was latterly preferred as the extracellular marker.

Estimation of Inulin and Raffinose in Extracts of Cardiac Muscle

Inulin and raffinose were estimated in deproteinised aqueous extracts of cardiac muscle. The size of the particles of dry tissue from which the extracts were made depended on the technique which was employed for the rapid inhibition of glucose metabolism. The material varied from a fine powder, when a frozen heart was disintegrated by ultrasonication, to pieces not greater than 1 mm in their maximum dimension, when a heart was minced with scissors.

Gilbert (1963) concluded from the percentage recovery of inulin and

raffinose from extracts of rat cardiac muscle that the hearts contained approximately 71 µg. of material which reacted as inulin and 200 to 300 µg. which reacted as raffinose. These values were subject to large variations and did not show any close correlation with the weight of the tissue. The higher "blank" in the case of raffinose clearly reflects the lower proportion of residues of fructose in this substance in comparison with inulin. The total amounts of the apparent cardiac inulin or raffinose, which were determined in this work, were reduced by the appropriate value for the blanks (71 µg. for inulin and 210 µg. for raffinose). Failure to make this correction would have resulted in an overestimate of the amount of cardiac inulin of not more than 1.5% and an overestimate of the amount of cardiac raffinose of not more than 5%. Because of the uncertainty of the values for the blank, inulin is clearly the preferable extracellular marker. However, the technique which appeared most effective in rapidly inhibiting glucose metabolism also resulted in a relatively large volume of extracellular water which contained as much as 7.5 mg. of raffinose. In this circumstance an error of 2.8% would be incurred by neglecting the blank. Despite the error in the blank, the estimate of cardiac raffinose would be of acceptable accuracy after the correction. A higher concentration of raffinose in the perfusate than the 1 g./100 ml. which was used in this work would allow more precise estimates of the cardiac raffinose, but would also increase the osmolarity of the medium by 20 mosmoles/l for a doubling of the amount of cardiac raffinose.

EFFECTIVENESS OF METHODS FOR RAPID INHIBITION OF GLUCOSE METABOLISM

All attempts to inhibit the metabolism of glucose were based on the principle of cooling the heart and denaturing the enzymes of the heart with ethanol. This approach was used with some success by Fisher and Lindsay (1956), when hearts were rapidly minced in ice-cold ethanol. To some extent the objectives of cooling and denaturation are antagonistic. Freezing a tissue prevents the penetration of ethanol or any chemical inhibitor of metabolism, but rapid cooling of a tissue is clearly favoured by a large temperature difference between the coolant and the tissue. In addition, the thermal conductivity of ice is greater than that of water. Thus an initially rapid rate of cooling is favoured by a final low temperature while the rapid penetration of ethanol into the tissues requires that the final temperature of the tissues is above the freezing-points of their liquid.

The treatment of the heart must also be compatible with the ultimate object of estimating the amount of cardiac glucose and thence, ideally, the concentration of intracellular glucose. It is, therefore, desirable to attempt to minimise the amount of extracellular water, and hence of extracellular glucose so that, in principle, the contribution of intracellular glucose to the total cardiac glucose might be measured more accurately. Determination of the weight of the heart when wet and when dry for the estimation of the total water of each heart is also useful for the measurement of the volume of intracellular water.

The first attempts to inhibit the metabolism of glucose, which were made in this work, were based upon the rapid exposure to ethanol of hearts which

TABLE 23

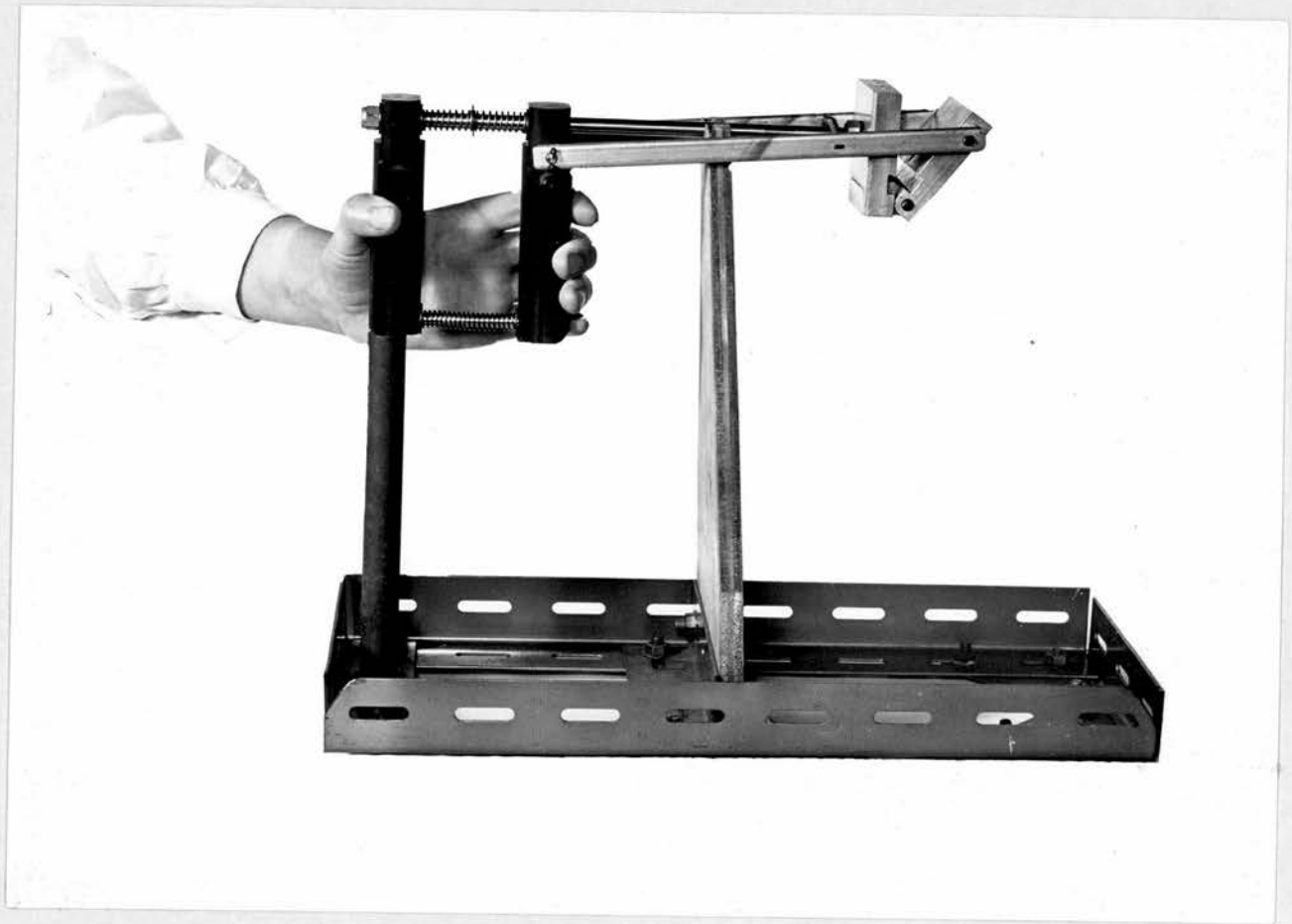
THE VOLUME OF DISTRIBUTION OF GLUCOSE IN HEARTS PERFUSED WITHOUT INSULIN

After perfusion, hearts were squeezed between pads of filter paper – to reduce the extracellular volume – and minced with scissors in ice-cold alcohol to inhibit metabolism. The column headed "Deficit" shows the difference between estimated amount of cardiac glucose and the theoretical amount which would give a glucose space equal to the volume of extracellular water. Raffinose was the extracellular marker when the perfusate glucose was less than 100 mg./100 ml. Otherwise inulin was the marker.

Extracellular water ml.	Glucose space ml.	Perfusate glucose concentration mg./100 ml.	Deficit µg.
0.384	0.378	283.5	17.6
0.355	0.348	184.6	13.0
0.379	0.361	135.2	24.1
0.389	0.368	132.7	27.3
0.366	0.343	126.0	29.8
0.359	0.337	91.8	20.6
0.471	0.444	91.1	34.1
0.369	0.339	85.9	26.4
0.397	0.378	76.1	14.7
0.441	0.406	43.9	15.1
0.459	0.281	40.7	72.5
0.577	0.470	30.7	32.8
0.495	0.347	23.0	34.4
0.561	0.370	18.9	36.2

were cooled but not frozen. Hearts were minced in ethanol at 0°C. In this early work, efforts were also made to minimise the volume of extracellular water. When hearts were transected and blotted after perfusion before they were exposed to the cold ethanol, the apparent glucose space was invariably considerably less than the extracellular space, although the procedure was performed as rapidly as possible. In a variant of this technique, the hearts, while still cannulated, were grasped between pads of filter paper and squeezed while the ventricular muscle was cut away from the remainder of the preparation. The ventricles were then transferred to ice-cold ethanol and minced. Values for the apparent glucose space in hearts which were treated by this technique always exceeded the extracellular space when insulin was included in the perfusate and the steady-state concentration of perfusate glucose was greater than 25 mg./100ml. Without insulin in the perfusate, no indication of the presence of intracellular glucose was observed, except when the hearts both used glucose and produced lactate at high rates. Values are given in Table 23, for the apparent glucose space, the extracellular space and the apparent deficiency of glucose in hearts which were perfused without insulin and produced little or no detectable lactate. It must be emphasised that the values in Table 23 for the glucose space have only qualitative significance so that the similarity of the apparent deficiency of cardiac glucose at all concentrations of perfusate glucose may not be meaningful. In all these experiments, the amount of cardiac glucose was determined with the "Acetate" reagent, which would be expected to result in an overestimate of the glucose space. Mincing in ice-cold ethanol therefore appears to be an inadequate technique for achieving the rapid inhibition of glucose

Fig. 33



The Wollenberger Clamp

metabolism in cardiac muscle.

It was argued earlier that if a tissue is frozen in an attempt to arrest metabolism, the final temperature of the tissue should be as low as possible to achieve an initially rapid rate of cooling, because the advantage of the penetration of the tissues by an inhibitor is lost in this circumstance. A commonly used technique for the rapid cooling of organs is the clamping of an organ between two blocks of aluminium which have been cooled in liquid nitrogen, - 195.8°C (Wollenberger, Riestau, and Schoffa, 1960). For this purpose, a device (Fig. 33) was constructed whereby two blocks of aluminium (6.2 cm. x 5.2 cm. x 1.4 cm.) could be forcefully opposed and which was operated with one hand. It was then possible to withdraw a perfused heart from the heart chamber and to clamp it while it was still being perfused. After compression, the frozen heart was approximately 2 mm. thick. The atria and aorta which protruded from the clamp were as far as possible broken off with special bone-forceps whose cutting edges could be opposed. After immersion in ethanol, in a container surrounded by a mixture of acetone and solid carbon dioxide, the ventricular muscle was fragmented with the bone forceps and further disrupted by ultrasonication, when the muscle was almost completely dispersed, leaving only one or two small pieces of what appeared to be largely connective tissue. The temperature of the ethanol after sonication was less than -5°C. After the mixture had been returned to room temperature, it was reduced to dryness in an apparatus for freeze-drying. Extracts of the dried tissue were then prepared in the manner which was described in the previous chapter.

This technique had the disadvantage that the volume of extracellular

TABLE 24

THE VOLUME OF DISTRIBUTION OF GLUCOSE IN HEARTS PERFUSED WITH INSULIN

After perfusion, hearts were clamped between aluminium blocks, cooled in liquid nitrogen. The frozen heart was disintegrated by ultrasonication in cold ethanol, reduced to dryness by freeze-drying, and extracted with water at 100° C. Raffinose was the extracellular marker.

Extracellular water ml.	Glucose space ml.	Perfusate glucose concentration mg./100 ml.
0.440	0.236	13.8
0.432	0.790	11.0
0.462	0.294	10.8
0.548	0.686	9.4
0.468	0.595	8.1
0.337	0.581	7.9
0.771	0.637	6.2
0.657	0.725	6.0

water could be as large as 0.8 ml. if in clamping the heart a drop of perfusate which was about to fall from the heart was also frozen. It was also impossible to determine the weight of the ventricular muscle when wet. Whereas there was no appreciable loss of weight of the ethanol container and its contents when a heart was minced in ethanol, sonication caused a loss of about 20 mg., which was also found when ethanol was sonicated in the absence of ventricular muscle. Consequently, the intracellular water could not be determined from the difference between total water and extracellular water. The volume of intracellular water may be estimated instead from its relationship with the weight of the dry cardiac tissues. Gilbert (1963) found the relationship to be constant, within the limits of experimental error, when the effect of a permeant on the osmolarity of the intracellular water is taken into account. However, an osmotic correction for glucose requires that the amount of intracellular glucose is known.

The assessment of the effectiveness of this technique in rapidly inhibiting glucose metabolism was limited largely to experiments in which hearts were perfused with insulin and at concentrations of perfusate glucose of less than 25 mg./100 ml. At these concentrations, the amount of extracellular glucose could not have exceeded 160 µg. and may have been as little as 20 µg. The apparent amount of glucose might therefore be at least trebled by material in the cardiac extracts which reacts as glucose with the "Acetate" reagent. Despite this, glucose spaces less than the extracellular volume were found in these conditions, as is shown in Table 24.

Rapid freezing of the heart does not therefore appear to be completely effective in inhibiting the metabolism of glucose. However, the possibilities

cannot be discounted that ultrasonication may have caused localised thawing of the frozen tissue or that extraction of glucose from the tissue may not have been complete despite the high degree of dispersion of the ventricular muscle. The suitability of techniques for the inhibition of metabolism will be discussed in the following chapter in the context of the problem of the determination of the concentration of intracellular glucose.

CHAPTER FOUR

DISCUSSION

The advantages which would be gained, for the purpose of this investigation, from the ability to make unexceptionable estimates of the concentration of intracellular glucose have been set out. The objective is to determine the distribution of the total cardiac glucose between the extracellular and intracellular compartments. Failure to achieve even the estimation of total cardiac glucose was attributed tentatively to the loss of glucose from solution during deproteinisation of extracts of cardiac muscle and therefore possibly to the choice of deproteinising agent. Zinc hydroxide was chosen because its use is compatible with the methods for the estimation of glucose, raffinose and, perhaps under rigorous conditions, of inulin. Metaphosphoric acid, which was the only commonly employed deproteinising agent which Gilbert (1963) found to be without interference in the estimation of inulin, was found in this work to affect the estimation of glucose.

There are some disadvantages in the obvious practice of making separate deproteinisations of aliquots of a cardiac extract for the two analyses. Accurate estimates of glucose utilisation, in the absence of insulin, could only be made when the concentration of perfusate glucose was less than approximately 100 mg./100 ml. Techniques were therefore sought which would allow the concentration of intracellular glucose to be determined over the greatest possible range of concentrations of perfusate glucose. Since a

heart which is exposed to a perfusate glucose concentration of 10 mg./100 ml. in the absence of insulin would be expected to contain only approximately 50 µg. of glucose and since the error in an estimate of glucose concentration is independent of the magnitude of the estimate, extraction and deproteinisation in a small final volume favours the accurate determination of total cardiac glucose at low concentrations of perfusate glucose. Any partition of the extract for the sake of the independent preparation of deproteinised samples for the estimation of glucose and an extracellular marker must reduce the accuracy of an estimate of total cardiac glucose.

However, qualitative investigations of the efficacy of techniques for the rapid inhibition of metabolism and the extraction of glucose suggested that the techniques were imperfect so that estimates of cardiac glucose might be irrelevant to the condition of a heart while perfused in a steady state. These investigations also underlined the difficulties in determining the distribution of the total cardiac glucose. The assumption of equality in the concentrations of perfusate and interstitial glucose is an approximation which must result in an overestimate of the amount of extracellular glucose and could introduce large errors in the determination of small amounts of intracellular glucose. Treatment of a heart after perfusion which maximises the contribution of intracellular glucose to the total cardiac glucose would appear to favour the accurate determination of intracellular glucose. However, the blotting of a heart, which in this work constituted such a treatment, must also increase the relative contribution of the interstitial glucose to the extracellular glucose and therefore increase an error which is dependent on a difference in concentration between perfusate and inter-

stitial glucose. Whatever may be the advantages of rapid freezing in inhibiting the metabolism of glucose, this technique reduces the relative contribution of intracellular glucose to the total cardiac glucose because of the uncontrollable freezing of perfusate on the surface of the heart in the act of clamping the hearts between aluminium blocks. The difficulties which this technique introduces in the determination of the volume of intracellular water were mentioned previously.

In conclusion, it is pertinent to consider the order of magnitude which may be expected of the concentration of intracellular glucose in the absence of insulin. The investigation into the kinetics of glucose utilisation, which is described in the following section, concludes that the maximum rate of glucose utilisation in the absence of insulin is about one-third of the Vmax for glucose phosphorylation in the presence of the hormone. The Km of glucose phosphorylation is estimated to be 0.5 mg/100 ml. Assuming that insulin does not influence the phosphorylation of glucose, the concentration of intracellular glucose in the absence of the hormone cannot exceed 0.25 mg./100 ml. that is, in a heart with 0.5 ml. of intracellular water, there would be no more than 1.25 µg. of intracellular glucose. This estimate would also be applicable to a heart which is perfused with insulin and at a concentration of perfusate glucose of 5 mg./100 ml. It is clear that existing techniques are inadequate for the determination of such small amounts of intracellular glucose.

CHAPTER FIVE

SUMMARY

1. The possibility of making accurate estimates of the concentration of intracellular glucose has been examined.

2. Determination of the distribution of the total cardiac glucose between the intracellular and extracellular water requires that no significant variations in glucose concentration occurs in the extracellular compartment. The effectiveness of the capillary wall as a barrier to the diffusion of glucose was considered and taken to be negligible.

3. Estimation of glucose in aqueous extracts of cardiac muscle, which were deproteinised with zinc hydroxide (a method compatible with the measurement of the extracellular volume) proved to be subject to large errors, even with a reagent of improved specificity for glucose.

4. Qualitative investigations of the effectiveness of methods for the rapid inhibition of metabolism and the subsequent extraction of glucose from cardiac muscle indicated that those used were inadequate.

5. It is concluded that precise estimates of the concentration of intracellular glucose, especially in hearts perfused without insulin at low concentrations of perfusate glucose, is impossible with the techniques presently available and used in this work.

SECTION IV

THE KINETICS OF GLUCOSE UTILISATION BY THE PERFUSED RAT HEART

CHAPTER ONE

INTRODUCTION

Inability to make unexceptionable measurements of the concentration of intracellular glucose made it impossible to determine the kinetics of glucose permeation in the isolated rat heart from the steady state relationship of the net inward transfer of glucose and the concentrations of extracellular and intracellular glucose. An indirect approach was therefore adopted.

The relationship between glucose utilisation and the concentration of extracellular glucose must reflect the kinetics of the transfer of glucose across the cell membranes and the kinetics of the intracellular metabolism of the sugar. The kinetics of glucose utilisation might approximate to those of transfer or those of the metabolism of glucose when in differing circumstances the rate of utilisation is limited by one or the other process. An analysis of the kinetics of glucose utilisation could reveal the parameters of the permeation process provided that the influence of the intracellular transformation of glucose can be taken into account.

Inward transfer may be assumed to be the only source of intracellular glucose because, in the absence of a glucose-6-phosphatase, the phosphorylation of glucose can be regarded as irreversible on account of the large negative change in free energy which accompanies the reaction. The possibil-

ity that α-amylases, detected in rabbit muscle (Abdullah, Taylor and Whelan, 1964), and α-glucosidase, present in rabbit cardiac muscle (Rosenfeld, Lukomskaya and Popova, 1966), may contribute to the formation of intracellular glucose was discounted. These enzymes are poorly characterised, but are of low total activity and, in vivo, may be separated spatially from their substrates. The kinetics of the metabolism of glucose may therefore be supposed to be those of the irreversible phosphorylation of glucose.

Since it is likely that, in the steady state, the rate of phosphorylation depends on a single saturable reaction, the assumption is made that the relation between the intracellular glucose concentration (y) and the rate of phosphorylation (v) is:

$$v = \frac{V_2 y}{K_2 + y} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where V_2 is the Vmax of phosphorylation

and K_2 is the Km of phosphorylation

According to the carrier hypothesis, the net rate of inward transfer of glucose, which in a steady state equals the rate of phosphorylation, is given by:

$$v = \frac{V_1 K_1 (x - y)}{(K_1 + x)(K_1 + y)} \qquad .. \quad .. \quad .. \quad .. \quad (2)$$

where x is the extracellular glucose concentration,

V_1 is the maximum rate of permeation

and K_1 is the half-saturation constant for permeation.

Substituting for y from (1) in equation (2) gives:

$$v = \frac{V_1 K_1 \left[x(V_2 - v) - K_2 v\right]}{\left[K_1 + x\right]\left[K_1 (V_2 - v) + K_2 v\right]} \qquad .. \quad .. \quad (3)$$

that is, if the assumptions embodied in equations (1) and (2) are valid, there should be a unique relation between the rate of glucose uptake in the steady state and the extracellular glucose concentration.

Two useful expressions can be derived from equation (3), which rearranges to:

$$K_1(K_1-K_2)v^2 + x(K_1-K_2)v^2 - K_1(K_1V_2+K_2V_1)v - K_1 x (V_1+V_2)v + V_1V_2K_1x = 0 \qquad (4)$$

Division of equation (4) by $vK_1(K_1-K_2)$ and rearrangement gives

$$v = \frac{K_1V_2+K_2V_1}{K_1 - K_2} + \frac{V_1+V_2}{K_1-K_2}\cdot x - \frac{V_1V_2}{K_1-K_2}\cdot\frac{x}{v} - \frac{1}{K_1}\cdot vx \qquad .. \quad .. \quad .. \quad (5)$$

Equation (5) has the merit that the half-saturation constant for permeation (K_1) is separated as the reciprocal of the coefficient of vx.

Alternatively, differentiation of equation (4) with respect to v and x and setting $dv/dx = 0$, gives:-

$$(K_1-K_2)v^2 - K_1(V_1+V_2)v + V_1V_2K_1 = 0 \qquad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

When $dv/dx = 0$, the rate of glucose utilisation in the coupled process of permeation and phosphorylation is a maximum. The applications which were made of equations (5) and (6) are discussed in the following chapter.

A further approximation can be made in addition to those implicit in the original assumptions. When the concentration of intracellular glucose is so low that the efflux of glucose is negligible in comparison with influx, equation (2) can be simplified to:

$$v = \frac{V_{K_1} x}{K_1 + x} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (7)$$

that is, the kinetics of glucose utilisation approximate to those of unidirectional permeation. The relation between the utilisation of glucose and the extracellular glucose concentration in hearts perfused without insulin might be expected to conform to equation (7). If insulin increases glucose uptake by promoting the transfer of glucose into the cells and does not influence the phosphorylation of glucose, the process of permeation must limit the utilisation of glucose in the absence of the hormone.

In the presence of insulin, permeation is unlikely to limit the utilisation of glucose at physiological concentrations of the extracellular sugar. In these conditions, intracellular glucose can be detected (Morgan, Henderson et al. 1961), and the efflux of glucose may be significant. However, at lower concentration of extracellular glucose, the intracellular concentration and the efflux of glucose might become negligible. Conformity of the relation between glucose utilisation and the extracellular concentration of glucose to equation (7) would then be observed at low concentrations of glucose but not at high concentrations. The relation which is described by equation (3) would be expected to hold at all concentrations of extracellular glucose. Evidence which supports this expectation will be given in the following chapter where estimates of the parameters of permeation and phosphorylation in the presence of insulin are also given.

Estimation of the parameters of permeation in the absence of insulin is the subject of the third chapter and in Chapter Four the accuracy and significance of the estimates and of the effect of insulin thereon are discussed.

CHAPTER TWO

THE PARAMETERS OF THE PERMEATION OF GLUCOSE IN CARDIAC MUSCLE THE PRESENCE OF INSULIN.

PROCEDURE

The utilisation of glucose by hearts was measured at constant concentrations of perfusate glucose when insulin was included in the perfusate at 100 mU/ml. The concentrations of glucose in the perfusate and the interstitial water were assumed to be equal. A high concentration of insulin (100 mU/ml.) was used to ensure that the concentration of insulin in the tissue fluid would almost immediately reach the level of about 2 mU/ml. necessary for maximal insulin effect on glucose uptake (Bleehen and Fisher, 1954). Morgan, Henderson et al. (1961) also used insulin at 100 mU/ml. and, in this respect, their results are comparable with those of this work.

The estimates of utilisation, from which the parameters of permeation are evaluated in this chapter, were obtained largely from analyses of the time-course of utilisation. In these instances, the estimates are averages of at least two and up to four measurements, which were made while the concentration of perfusate glucose was constant. Other estimates are the means of three measurements which were made when three successive fractions were collected during a 15 minute period of constancy in the concentration of glucose.

Estimates of utilisation have been expressed in terms of mg. per g. "True solid" per hour and not per unit of dry weight. The "True solid" weight is a correction of the dry weight of a heart made by subtraction of the weight of solids which, from the constitution of the perfusate, may be calculated to be present in the extracellular water of the heart. This correction eliminates variations in the dry weight between hearts, which are due to differences in the volume of extracellular water or in the constitution of the perfusate. Differences in the concentration of perfusate glucose caused variations in the weight of extracellular glucose of no more than 1 mg. in hearts whose dry weights fell in the range 130 to 170 mg. However, the results to be presented were obtained with hearts which were perfused with inulin (2% $^{w}/_{v}$) or raffinose (1% $^{w}/_{v}$) included in the perfusate. Difference in the dry weight of approximately 5 mg. could result from the choice of extracellular marker. The correction for the weight of electrolytes, glucose and extracellular marker in the extracellular water amounted to 7.0 ± 1.2% of the dry weight. When, on occasions, the estimation of the volume of extracellular water was not possible, as, for example, when the inulin was lost on deproteinisation, the dry weight was reduced by 7% to give an estimate of the true solid weight. The error inherent in the correction did not significantly increase the final error in the estimate of utilisation. This correction may have increased the error in an individual estimate, but provides a better basis for the comparison of results.

TABLE 25

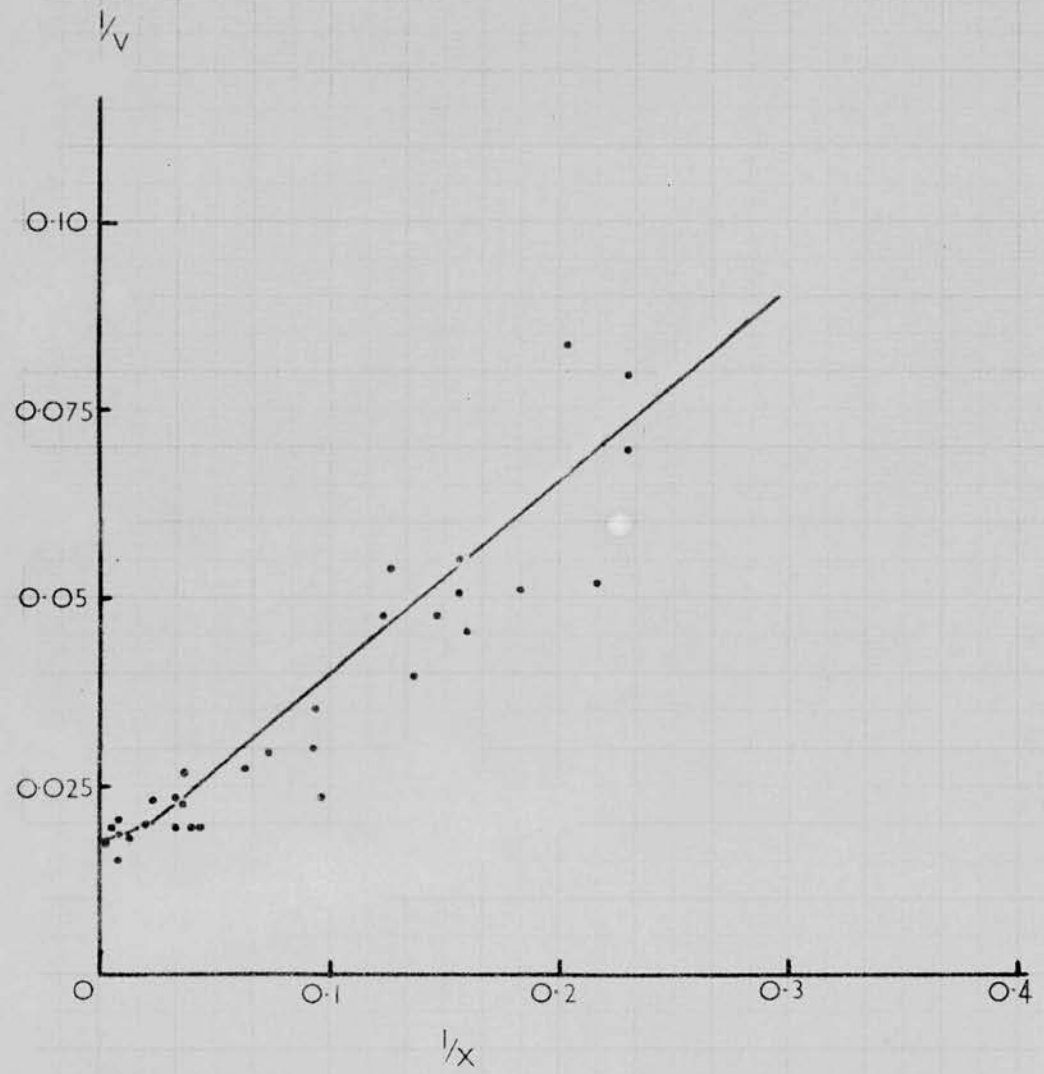
THE EFFECT OF GLUCOSE CONCENTRATION ON GLUCOSE UTILISATION BY THE RAT HEART PERFUSED WITH INSULIN (100 mU./ml.)

Perfusate glucose concentration mg./100 ml.	Glucose utilisation mg./g. true solid/hour	Perfusate glucose concentration mg./100 ml.	Glucose utilisation mg./g. true solid/hour
263.8	56.4	10.9	28.7
165.8	50.2	10.8	33.1
125.4	49.4	10.6	42.8
123.3	53.0	8.1	21.0
114.9	66.5	7.9	18.6
72.3	55.0	7.4	25.2
49.4	49.9	6.7	21.1
35.1	43.6	6.3	18.2
31.1	43.0	6.3	19.7
29.5	51.5	6.2	22.0
27.8	44.8	6.0	22.3
27.1	37.1	5.5	19.6
24.7	51.0	4.9	11.4
24.3	50.5	4.6	19.4
15.8	36.7	4.4	14.4
13.8	33.7	4.4	12.5

Figure 34

EFFCT OF CONCENTRATION ON GLUCOSE UTILISATION

A Double-reciprocal Plot of Utilisation (V, mg./g true solid/hour) versus Glucose Concentration (X, mg/100 ml) in the Presence of Insulin (100 mU/ml)

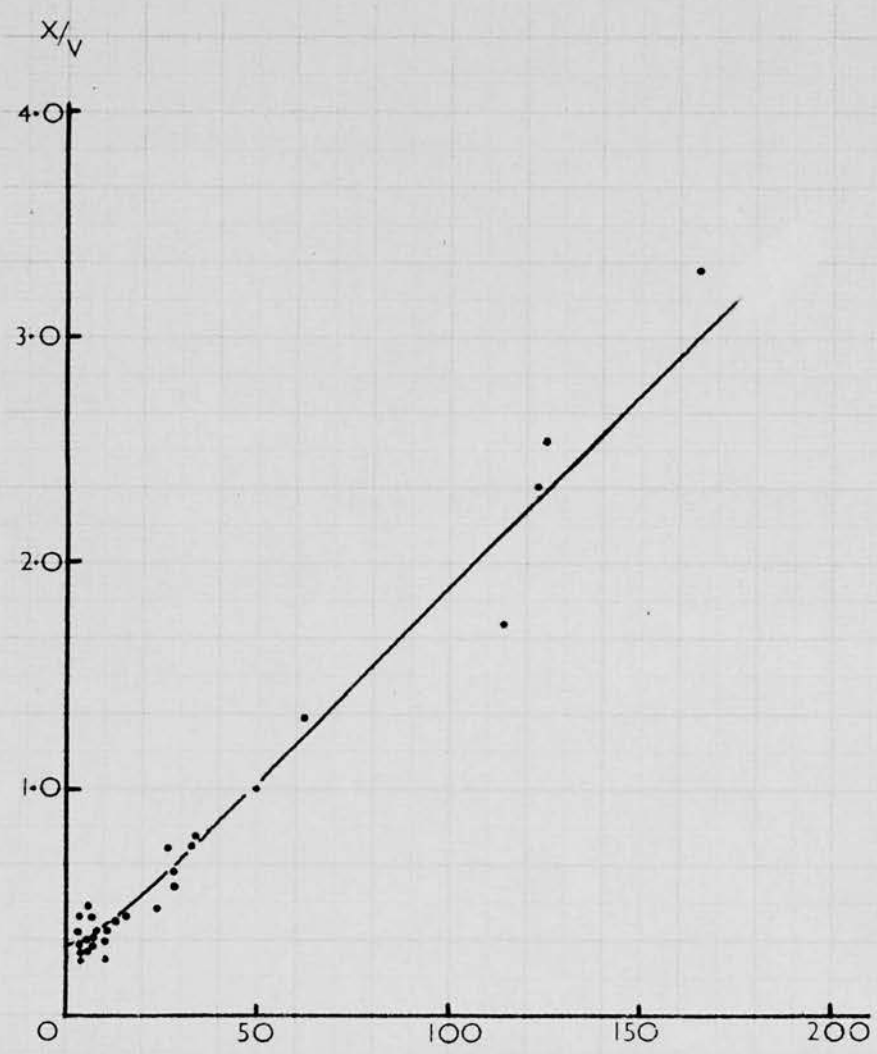


Each point is the result of one perfusion. The line through the points is the relationship predicted for a system of carrier-mediated permeation and irreversible phosphorylation where K_1 = 20 mg. /100 ml., V_1 = 80 mg./g. true solid/hour, K_2 = 0.5 mg. /100 ml. and V_2 = 60 mg./g. true solid/hour. The symbols have the significance ascribed in the text.

Figure 35

EFFECT OF CONCENTRATION ON GLUCOSE UTILISATION

A Plot of the Ratio of Perfusate Glucose Concentration (X mg./100 ml.) to Glucose Utilisation (V mg./g. true solid/hour) versus that Concentration



Each point is the result of one perfusion. The line through the points is the relationship predicted for a system of carrier-mediated permeation and irreversible phosphorylation where K_1 = 20 mg./100 ml., V_1 = 80 mg./g. true solid/hour, K_2 = 0.5 mg./100 ml., and V_2 = 60 mg./g. true solid/hour. The symbols have the significance ascribed in the text.

TABLE 26

COEFFICIENTS OF ASSUMED LINEAR RELATIONSHIPS BETWEEN THE RECIPROCALS OF GLUCOSE UTILISATION AND PERFUSATE GLUCOSE CONCENTRATION IN THE PRESENCE OF INSULIN

Range of glucose concentration	Slope and standard deviation**	Intercept and standard deviation**	Apparent Km mg./100 ml.	Apparent V_{max} mg./g. true solid/hour
All data of Table ~~24~~ 25	0.239 ±0.018	0.014 ±0.002	17.2	72.0
Data of Table ~~24~~ 25 > 20 mg./100 ml.	0.115 ±0.049*	0.018 ±0.001	6.5	56.6
Data of Table ~~24~~ 25 < 20 mg./100 ml.	0.283 ±0.044*	0.007 ±0.007	42.9	151.8

* These values differ significantly at the 5% level of probability.

** Units of concentration: mg./100 ml.; units of utilisation: mg./g. true solid/hour.

RESULTS

Estimates of the rate of utilisation of glucose by hearts which were perfused at a constant concentration of perfusate glucose, in the presence of insulin at 100 mU/ml., are given in Table 25. These results are plotted in Fig. 34 in the form of reciprocals. The relationship is not linear and, therefore, does not conform to equation (7) (p.151). In Fig. 35 the results of Table 25 are presented in the alternative manner of Lineweaver and Burk, where $^{x}/_{v}$ is plotted against x. Non-linearity is less evident in this presentation. The lines drawn through the experimental data are relations predicted from equation (3) (p.149).

A comparatively crude analysis of the data can be made by arbitrarily separating the data into two groups in which the concentration of perfusate glucose is greater or less than 20 mg./100 ml. The two groups were then treated as though they conformed to equation (7). Estimates of the apparent Km and Vmax for the two groups were obtained from the coefficients of the unweighted linear regressions values of $^{1}/_{v}$ and $^{1}/_{x}$. The results of these calculations are given in Table 26. A difference significant at the 5% level of probability exists between the values for the slopes.

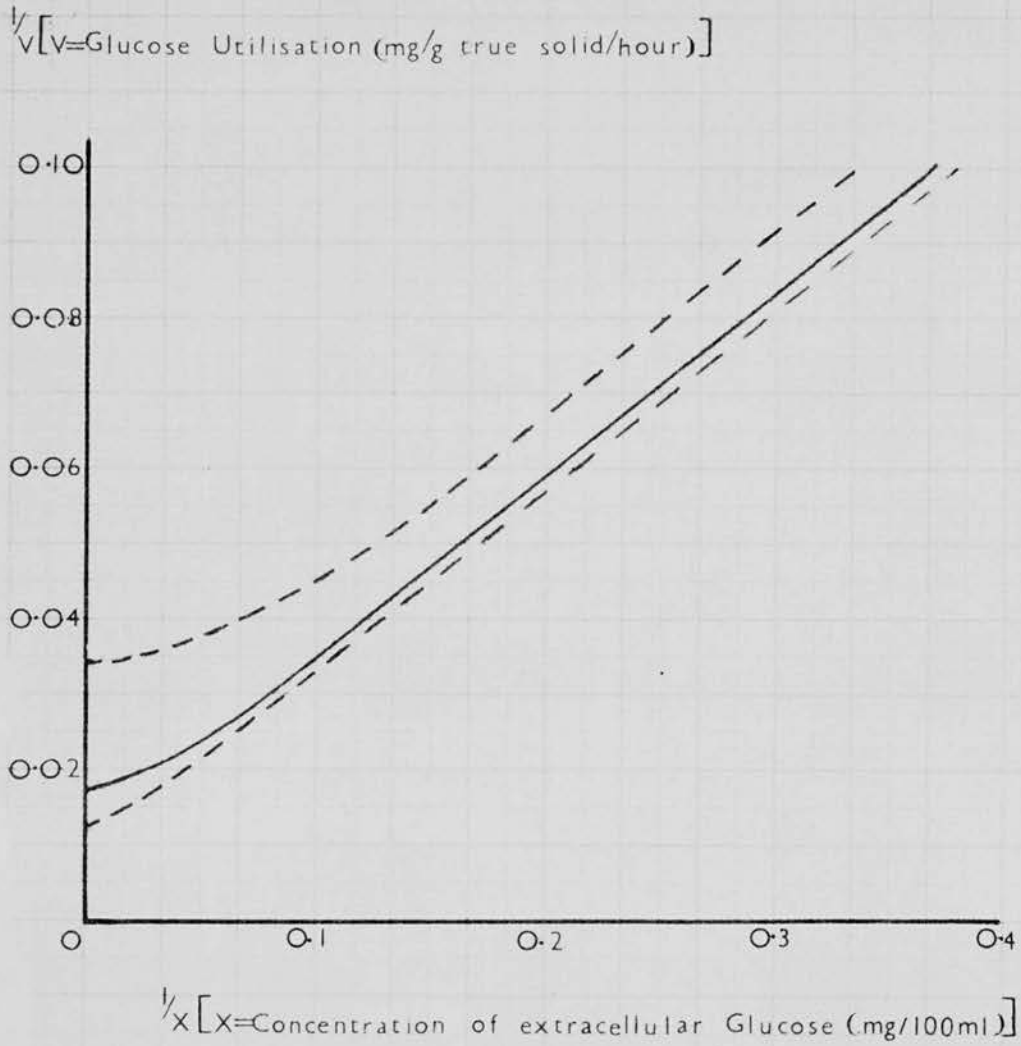
Large errors were associated with the values for the apparent Km and Vmax for the experimental observations which were made when the concentration of perfusate glucose was less than 20 mg./100 ml. However, the Vmax, 57 mg./g. true solid/hour, which was found at higher concentrations of perfusate glucose, had a comparatively small error.

The maximum rate of utilisation which is attainable in a system of rever-

Figure 36.

PROPERTIES OF A MODEL OF GLUCOSE UTILISATION

The Effect of Variations in the Vmax. of Phosphorylation

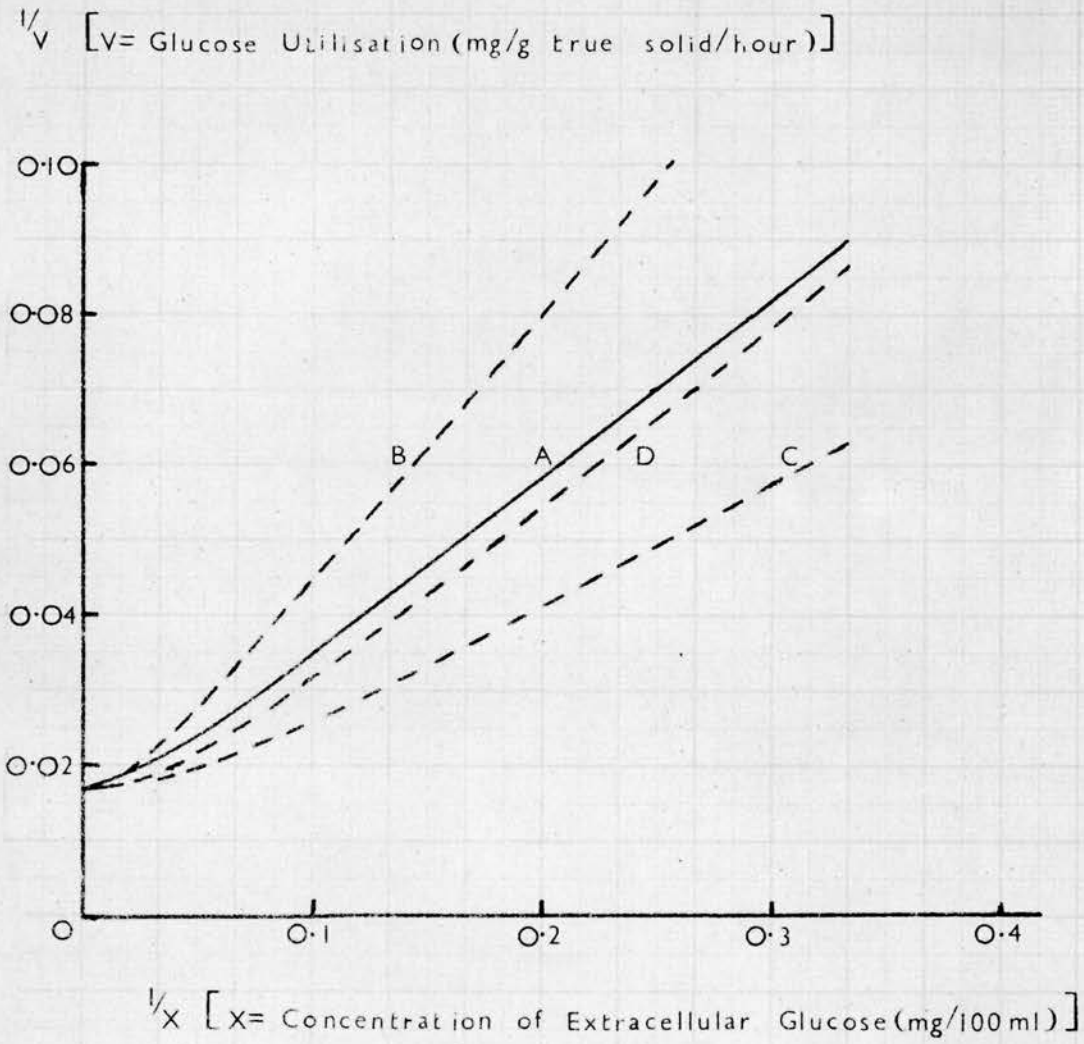


The model assumes carrier-mediated permeation and irreversible phosphorylation. The continuous line is given when K_1 = 27 mg./100 ml., V_1 = 120 mg./g. true solid/hour, K_2 = 0.9 mg./100 ml. and V_2 = 60 mg./g. true solid/hour. The effects of a 50% decrease and of a 50% increase in V_2 are shown by the upper and lower interrupted lines respectively. The significance of the symbols is given in the text.

Figure 37.

PROPERTIES OF A MODEL OF GLUCOSE UTILISATION

The Effect of Variations in the Parameters of Permeation



The model assumes carrier-mediated permeation and irreversible phosphorylation. The continuous line A is given when K_1 = 27 mg./100 ml., V_1 = 120 mg./g. true solid/hour, K_2 = 0.9 mg./100 ml. and V_2 = 60 mg./g. true solid/hour. The interrupted lines B and C show the effects of 50% increases in K_1 and V_1 respectively. A simultaneous 50% increase in K_1 and V_1 gives the line D. The significance of the symbols is given in the text.

sible carrier-mediate permeation and irreversible phosphorylation is given by equation (6) (p.150). An exploration was undertaken of the possible combinations of K_1, K_2, V_1 and V_2 which give a maximum rate of utilisation of the system of approximately 57 mg./g. true solid/hour. This exploration was complemented by an investigation of the relationship between $^1/v$ and $^1/x$ defined by Eqn. (3) (p.149) when the chosen values for the constants satisfied the required value for the maximum rate of the system. This calculation was programmed for the Programma 101. Although V_1 and V_2 in equation (6) are clearly interchangeable, in equation (3) they are not. A comparison of the relationship between $^1/v$ and $^1/x$, which was derived from the model system, with the experimental observations of Fig. 34 enabled V_1 and V_2 to be distinguished. The model system (equation (3)) predicts a non-linear relationship between $^1/v$ and $^1/x$, which does, however, tend towards linearity when x is not greater than K_1. The slope of the linear part of the relationship approximated to K_1/V_1.

A relationship between $^1/v$ and $^1/x$ which shows satisfactory conformity to the experimental data of Fig. 34 is obtained when K_1 = 27.0 mg./100 ml., K_2 = 0.9 mg./100 ml., V_1 = 120 mg./g. true solid/hour and V_2 = 60 mg./g. solid/hour. When alterations of 50% in the values of K_1, V_1 or V_2 were made without changing the values of the other constants, the conformity to the experimental observations was considerably reduced (Figs. 36 and 37). Alterations in K_2 of this order had little effect on the relationship. Although, when K_1 or V_1 were altered alone, there was a marked effect on the theoretical relationship of $^1/v$ and $^1/x$, the effect was largely nullified when proportional changes were made in K_1 and V_1 together. Estimation of K_1

TABLE 27

COEFFICIENTS OF A MULTIVARIATE EQUATION RELATING GLUCOSE UTILISATION AND GLUCOSE CONCENTRATION

$$v = \frac{K_1 V_2 + K_2 V_1}{K_1 - K_2} + \frac{V_1 + V_2}{K_1 - K_2} \cdot x - \frac{V_1 V_2}{K_1 - K_2} \cdot \frac{x}{v} - \frac{1}{K_1} \cdot vx$$

K_1 and V_1 are the parameters of glucose permeation.

K_2 and V_2 are the parameters of glucose phosphorylation.

v is the rate of glucose utilisation.

x is the concentration of perfusate glucose.

Coefficient	Value
$\frac{K_1 V_2 + K_2 V_1}{K_1 - K_2}$	46.9 ± 2.3
$\frac{V_1 + V_2}{K_1 - K_2}$	5.4 ± 0.6
$\frac{V_1 V_2}{K_1 - K_2}$	164.0 ± 17.0
$\frac{1}{K_1}$	0.043 ± 0.006

The coefficients were calculated by multiple regression, from the data of Table 25, by the method of Snedecor (1946a).

and V_1 therefore required the independent determination of one of them.

K_1 is separated from V_1 in the equation (5) (p. 150).

$$v = \frac{K_1V_2 + K_2V_1}{K_1 - K_2} + \frac{V_1 + V_2}{K_1 - K_2} \cdot x - \frac{V_1V_2}{K_1 - K_2} \cdot \frac{x}{v} - \frac{1}{K_1} \cdot vx \quad .. \quad .. \quad (6)$$

If a multiple regression of v on x, x/v, and vx is fitted, the coefficient of vx is an estimate of $1/K_1$. I am grateful to Mr. I.A. Nimmo, who developed a programme for the Programma 101 for the calculation of the coefficients of the multiple regression equation from the experimental data of Table 25. The values for the coefficients of equation (3) are given in Table 27. From this analysis, K_1 was found to be 23.0 ± 3.2 mg./100 ml., which is in reasonable accord with the value estimated by the exploratory fitting of curves.

The estimates of the values of the other coefficients of the regression equation provide an indication of the order of V_1, V_2 and K_2. Fig. 36 shows that V_2 is the main determinant of the maximum rate of utilisation in the model system so that 60 mg./g. true solid/hour may be taken as a reasonable estimate of V_2. If K_2 is assumed to be negligible in comparison with K_1 in the coefficients $\frac{V_1+V_2}{K_1-K_2}$, and $\frac{V_1V_2}{K_1-K_2}$, when K_1 is taken to be 23.0 ± 3 and V_2 to be 60, the maximum value for V_1 which is compatible with the computed values for the coefficients, is 80 mg./g. true solid/hour. The lines which have been drawn through the experimental data of Figs. 34 and 35 were computed for the model system when K_1 = 20 mg./100 ml., V_1 = 80 mg./g. true solid/hour, K_2 = 0.5 mg./100 ml., and V_2 = 60 mg./g. true solid/hour. This treatment indicates that the probable values for K_1 and V_1 are approximately 25% less than those which were obtained by the exploratory fitting of curves

to the experimental data.

That is, this analysis suggests that in the presence of maximal insulin the values of the parameters of glucose utilisation are:

K_1 20 mg./100 ml., and

V_1 80 mg./g. true solid/hour for the permeation of glucose;

K_2 0.5 mg./100 ml., and

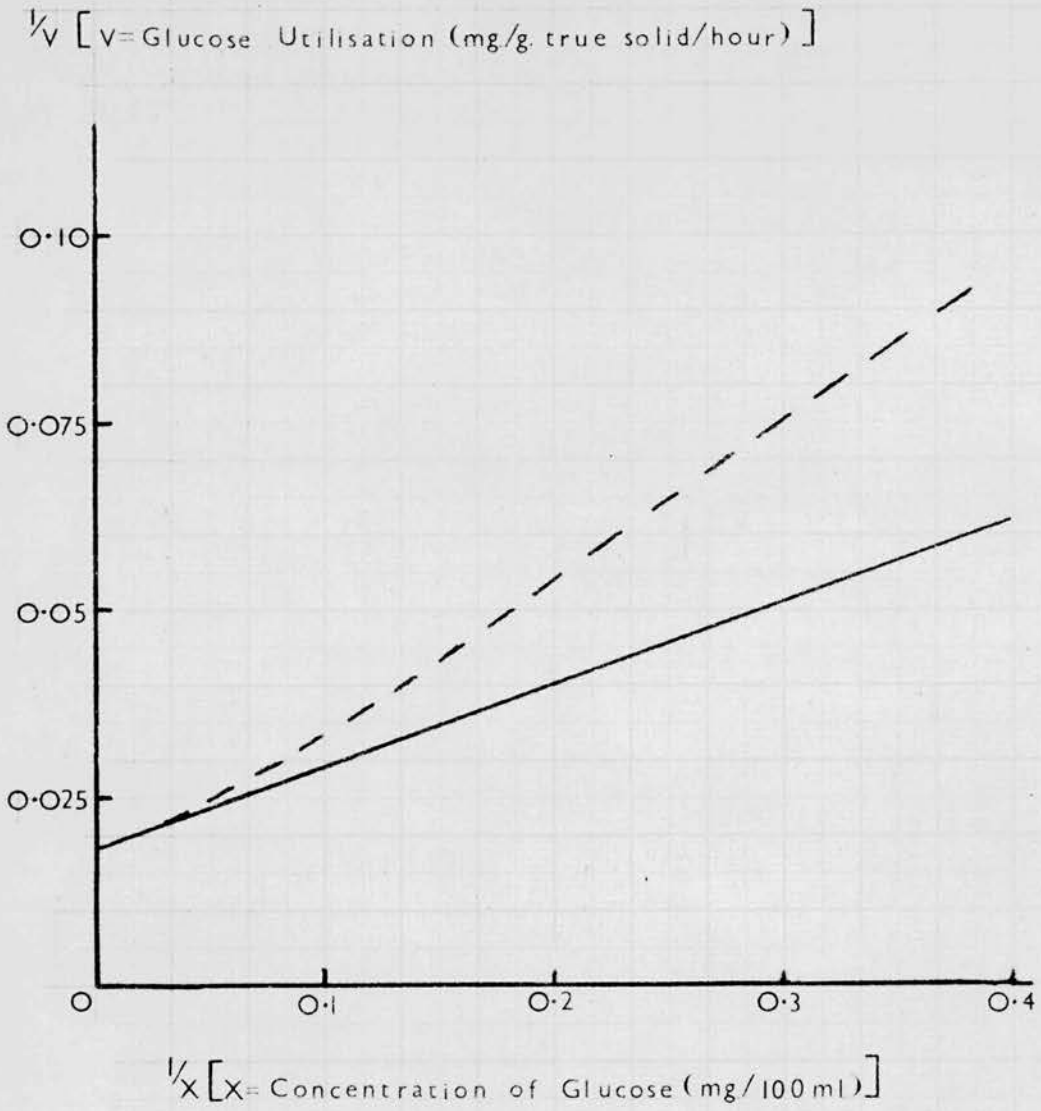
V_2 60 mg./g. true solid/hour for the phosphorylation of glucose.

DISCUSSION

The interpretation of the experimental results which has been adopted depends on the validity of the assumption that, in the steady state, the difference in concentration of interstitial and perfusate glucose is negligible. It is evident from the non-linear relationship between the concentration of perfusate glucose and the utilisation of glucose that the difference between the concentrations of perfusate and interstitial glucose must be greater, in relation to their magnitudes, at low concentrations than at high. The curvilinear relationship between $^1/v$ and $^1/x$ (Fig. 35), where x is the concentration of perfusate glucose, might therefore be supposed to reflect an increasing overestimate of the concentration of interstitial glucose as the concentration of perfusate glucose decreases. Although the reality of a concentration difference between perfusate and interstitial glucose is undoubted, the order of magnitude of the difference, which was discussed in the introductory chapter to the preceding section, is uncertain, but quite possibly insignificant in the present context.

Figure 38

Effect of the Spatial Separation of the Sites where a Substrate is Estimated and Transformed on the Form of a Double reciprocal Plot



The reciprocals of the data in Table 25 in which the concentration of perfusate glucose exceeded 20 mg./100 ml. are related by the regression coefficients:-

$$1/V = 0.0177 + 0,1152.\ 1/X$$

This relationship is plotted as a continuous line. The effect of increasing all values of X by an amount 0.1 times the corresponding values for V is shown by the interrupted line.

The coefficients of a linear regression equation were calculated for the data of ~~Fig. 35~~ Table 25 when the concentration of perfusate glucose was higher than 20 mg./100 ml. This equation was then used to calculate the concentration of glucose appropriate to the observed rate of utilisation for the remaining data, where the concentration was less than 20 mg./100 ml. In all but one of 18 cases, the predicted concentration was lower than the observed. The differences between the predicted and the observed concentrations varied between 1.6 and 4.7 mg./100 ml. and, expressed as a percentage of the observed concentration, ranged from 15 to 66% (Mean 40.4 ± 14.3%). Despite the arbitrary selection of data and the assumption of linearity, these calculations provide an indication of the magnitude of the difference in concentration between interstitial and perfusate glucose whose existence might explain the experimental observations.

Fig. 38 illustrates the form of a plot of the reciprocals of glucose utilisation and the concentration of glucose, when the rate of utilisation is limited in part by the rate of diffusion of the sugar to the enzyme. The plot was constructed by calculating, from the coefficients of the linear regression equation for the reciprocals of the experimental observations when the concentration of glucose exceeded 20 mg./100 ml., values for the concentration of "interstitial glucose" for particular rates of utilisation. These values were corrected to give the concentration of "perfusate glucose" by the addition of a constant multiple of the rate of utilisation, - 0.1 mg./100 ml. for every mg. of glucose metabolised per hour. The form of the plot clearly indicates that the experimental observations might reflect the influence of an increase in the relative difference between the concentrations

of interstitial and of perfusate glucose with decreasing concentration, but without knowledge of the order of magnitude of the differences, their significance cannot be assessed.

If it were supposed that both carrier-mediated permeation of cardiac cells and transcapillary diffusion influenced the experimental observation, it must be concluded that the values which have been given for the parameters of permeation are erroneous because the effect of an extracellular gradient in glucose concentration is to increase the slope of a plot of $^1/v$ against $^1/x$ when x is low. Fig. 34 shows that a slope less than that observed requires that V_1 is increased, K_1 is reduced or both. In the final chapter of this section, it will be suggested, from consideration of the parameters of permeation in the absence of insulin and the concentration-dependent stimulation of glucose utilisation by endogenous insulin, that a higher value for V_1 is more reasonable than a lower value for K_1.

CHAPTER THREE

THE PARAMETERS OF THE PERMEATION OF GLUCOSE IN CARDIAC MUSCLE IN THE ABSENCE OF INSULIN

INTRODUCTION and METHODS

In a steady state, when the kinetics of the utilisation of glucose by the isolated heart are assumed to approximate to those of permeation, conformity to equation (7) p.151 is predicted.

The utilisation of glucose by isolated rat hearts, which were perfused without added insulin, was measured over a range of concentration of perfusate glucose from approximately 3 to 300 mg./100 ml. Again, mg. of glucose/g. true solid weight/hour were the units chosen to express the rate of utilisation. When an extracellular marker was included in the perfusate, the true solid weight of the tissues was determined by the correction of the dry tissues in the fashion which was described on p.153. However, in some experiments, an extracellular marker was omitted. In these cases, after perfusion, adherent perfusate was blotted from the bisected heart and the remaining volume of extracellular water, whose dissolved solids would contribute to the dry weight, was calculated from its relationship with the weight of blotted cardiac muscle, which has been found to be approximately 0.35 ml. of extracellular water per g. wet tissue (Fisher and Young, 1961;

TABLE 28

THE EFFECT OF GLUCOSE CONCENTRATION ON GLUCOSE UTILISATION BY THE RAT HEART PERFUSED WITHOUT INSULIN

Group		Individual results						Means and standard deviations
1	Glucose concentration	95.2	93.1	91.8[X]	91.1			92.8 ± 1.8 mg./100 ml.
	Glucose utilisation	6.1 b	11.6	23.7 abc	19.9 abc			15.3 ± 8.0 mg./g. true solid/hour
2	Glucose concentration	48.1	43.9	42.3	41.0	40.9[X]	40.7	42.8 ± 2.9 mg./100 ml.
	Glucose utilisation	5.6	19.0 abc	15.1	15.3	18.8	24.2 abc	16.3 ± 6.2 mg./g. true solid/hour
3	Glucose concentration	39.7[X]	35.4[X]	34.4[X]	34.2[X]			35.9 ± 2.6 mg./100 ml.
	Glucose utilisation	16.3	25.4	27.6	28.4 bc			24.4 ± 5.6 mg./g. true solid/hour
4	Glucose concentration	30.7	29.3[X]	29.2[X]	27.8[X]	25.8	24.9[X]	28.0 ± 2.2 mg./100 ml.
	Glucose utilisation	20.3 bc	30.4	8.4	10.6	26.9 bc	24.3 bc	20.1 ± 8.9 mg./g. true solid/hour
5	Glucose concentration	(23.5)	23.0[X]	20.7[X]	20.2[X]	20.1		21.0 ± 2.4 mg./100 ml.
	Glucose utilisation	(1.7) b	3.5 bc	21.4 bc	19.5 bc	8.0 b		13.1 ± 8.7 mg./g. true solid/hour
6	Glucose concentration	18.9[X]	18.5	17.7	17.6	15.5	15.0	17.2 ± 1.6 mg./100 ml.
	Glucose utilisation	8.2 bc	12.2 bc	14.0 bc	8.5	11.5	18.7	12.2 ± 3.9 mg./g. true solid/hour
7	Glucose concentration	13.2	13.1	13.0	12.5	11.5		12.6 ± 0.7 mg./100 ml.
	Glucose utilisation	4.5	15.0	15.5	6.7	2.6		8.9 ± 6.0 mg./g. true solid/hour

TABLE 28 (cont.)

Group		Individual results					Means and standard deviations
8	Glucose concentration	10.5	9.7	9.4	9.0		9.6 ± 0.6 mg./100 ml.
	Glucose utilisation	9.5	5.1	11.6	12.5		9.7 ± 3.3 mg./g. true solid/hour
9	Glucose concentration	7.8^{x}	7.6	7.2	7.1	6.9^{x}	7.3 ± 0.4 mg./100 ml.
	Glucose utilisation	10.4	9.6	10.5	4.8	5.7_{b}	8.2 ± 2.7 mg./g. true solid/hour
10	Glucose concentration	6.4	6.4	5.9	5.4		6.0 ± 0.5 mg./100 ml.
	Glucose utilisation	5.7	7.6_{b}	8.7	10.6		8.2 ± 2.1 mg./g. true solid/hour
11	Glucose concentration	4.8	4.7	4.0	3.8		4.3 ± 0.5 mg./100 ml.
	Glucose utilisation	6.5_{b}	7.8	7.9	7.0_{b}		7.3 ± 0.7 mg./g. true solid/hour

Notes:

1. Subscripts indicate variations from a standard condition of perfusion in a polyvinyl heart chamber with anti-insulin serum (AIS) in the perfusate and the time-course of utilisation being determined. Variations are:—
   a. Durex rubber heart-chamber.
   b. No AIS in perfusate.
   c. Time-course of utilisation not followed.
2. Superscript x denotes detectable lactate formation, i.e. rate greater than 1 mg./g. true solid/hour.

Morgan, Henderson, et al., 1961). Without an extracellular marker, the weight of extracellular solids was no greater than 4 mg. in a dry heart of 150 mg. so that the error inherent in the correction would not greatly affect the accuracy of the estimate of the true solid weight. The units which have been chosen provide an adequate basis for the comparison of estimates of glucose utilisation.

The results of those experiments in which the concentration of perfusate glucose exceeded, in a steady state, 100 mg./100 ml. will be omitted from the subsequent analysis, because, as was discussed on p.106, the errors in the majority of estimates of glucose utilisation exceeded 10%. This upper limit to the range of concentration of perfusate glucose excludes all but one of the results with errors greater than 10% from the remaining data, which are presented in Table 28. That one result, which is given in parentheses in Group 5, has also been omitted from all analyses, not only because of the large error associated with it, but also because, in comparison with all other experiments, the rate of glucose utilisation was anomalously low.

All values in Table 28 for the utilisation of glucose were measured in a steady state of glucose metabolism after 60 to 90 minutes of perfusion. However, the results were obtained under conditions in which the concentration of perfusate glucose and the presence or absence of an extracellular marker were not the only variables between experiments. These variations are indicated in Table 28. Anti-insulin serum was included in the perfusate to which many hearts were initially exposed. Because the serum was not present in the infusate, its concentration in the recirculating perfusate must have decreased exponentially with a half-time of 7.5 minutes.

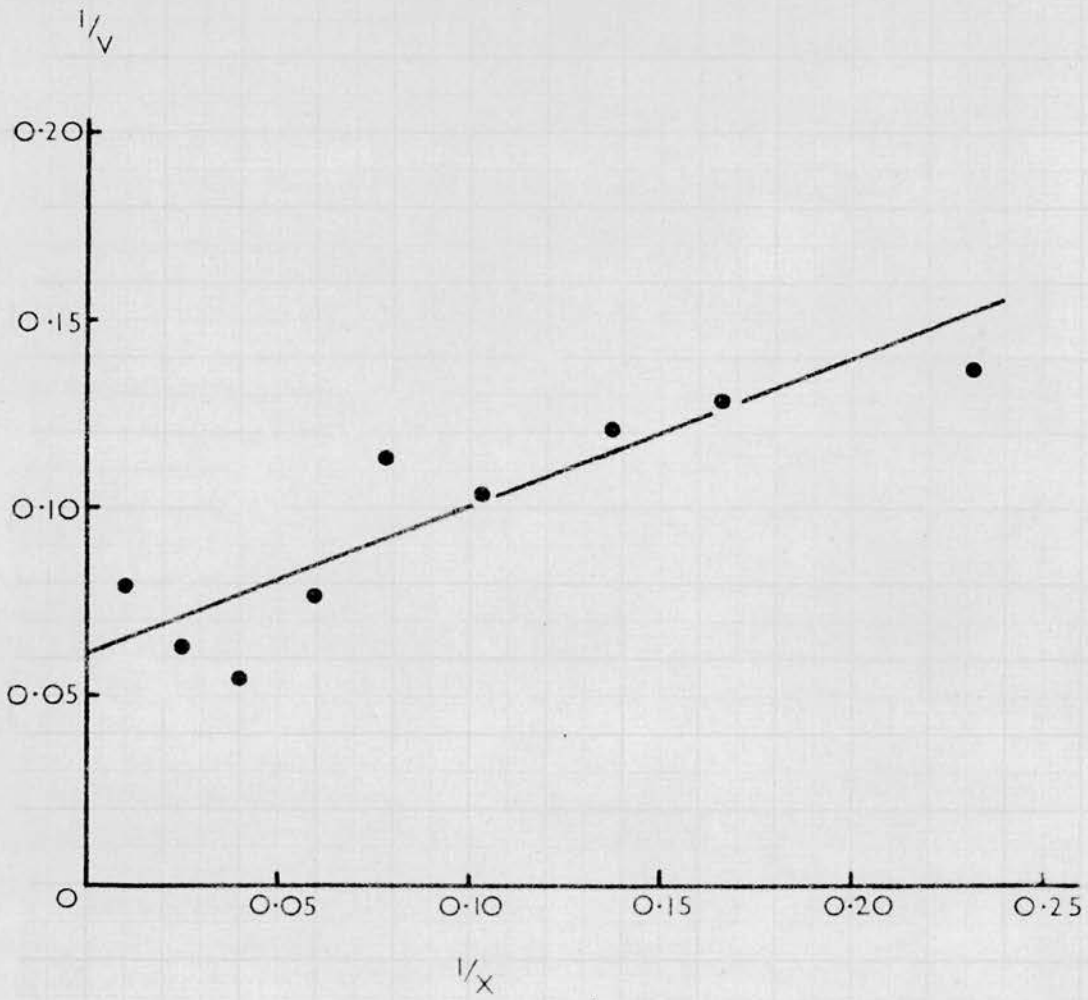
Minor changes were made in the perfusion apparatus as well as in the perfusate. Although in the majority of experiments, the hearts were enclosed in a polyvinyl chamber, on occasion the heart chamber consisted of the teat portion of an unlubricated Durex contraceptive. In all instances, the perfusate was passed through a Millipore filter. There was no evidence to suggest that the material of the heart chamber or the presence of anti-insulin serum affected the properties of the preparation. All experiments in which the heart beat irregularly, became hypodynamic or produced more than 10 mg. of lactate/g. true solids/hour were discarded.

It was argued in Section I (p.57) that the rate of formation of lactate by an isolated, perfused heart may be used as a measure of the excellence of the preparation. Many technical failings in cardiac perfusion can be expected to lead to local or general hypoxia and consequently a high rate of lactate formation through, for example, inadequate filtration of the perfusate which might result in blockage of the coronary vessels, or inadequate oxygenation of the perfusate. While gross deficiencies in a preparation are easily recognised and an experiment therefore disregarded, minor deficiencies, which might be associated with only small increases in the rate of lactate formation, cannot be so readily detected nor the results so confidently set aside. In an attempt to give greater weight to the experiments in which hearts were less likely to have suffered undetected maltreatment, the data of Table 28 have been analysed, not only as a whole, but also after the elimination of results from all experiments in which the formation of lactate was detectable. In this work, the lowest detectable rate of lactate formation was approximately 1 mg./g. true solid/hour.

Figure 39

EFFECT OF CONCENTRATION ON GLUCOSE UTILISATION

A Double-reciprocal Plot of Utilisation (V, mg/g true solid/hour) versus Glucose Concentration (X, mg/100ml) in the Absence of Insulin



The points are the reciprocals of the mean values in Table 29. The line is given by the model of carrier-mediated permeation and irreversible phosphorylation when K_1 = 6.5 mg./100 ml., V_1 = 17.0 mg./g. true solid/hour, K_2 = 0.5 mg./100 ml. and V_2 = 60.0 mg./g. true solid/hour. The significance of the symbols is given in the text.

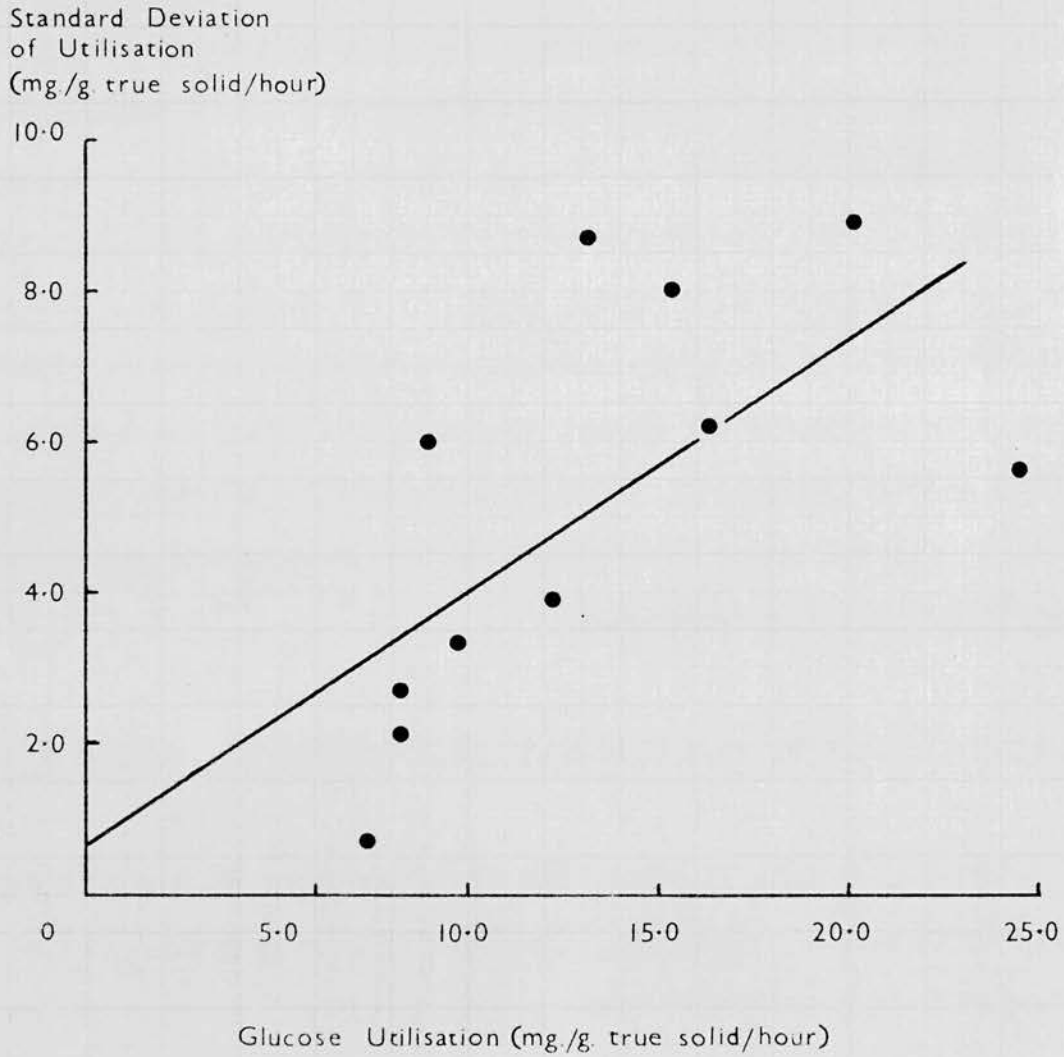
The data of Table 28 have been separated into groups. Grouping is convenient for graphical presentation and also permits the estimation of the variance of an individual result. Thus in Fig. 39, the reciprocals of the means of these groups (after elimination of those hearts producing detectable lactate (see Table 29)) are plotted. At the higher concentrations of perfusate glucose, the grouping was determined by the concentration of infusate glucose, which was the same in a series of experiments so that similar concentrations of perfusate glucose were established in the steady state when hearts of about the same weight were perfused. However, at lower concentrations of perfusate glucose, the basis of the grouping was less simple. Experiments were designed so that the steady state concentrations of perfusate glucose would give an approximately uniform spread on the abscissa of a double-reciprocal plot. At low concentrations, the ranges of the steady state concentrations of perfusate glucose established at each concentration of infusate glucose overlapped. In these circumstances, the data have been grouped so that the standard deviations of the means of the concentrations of perfusate glucose are minimised.

ESTIMATES OF THE PARAMETERS OF GLUCOSE UTILISATION IN THE ABSENCE OF INSULIN

Inspection of Table 28 shows that the variability of the estimates of glucose utilisation at comparable concentrations of perfusate glucose is considerable. From Bartlett's test of the homogeneity of variance (as used by Snedecor, 1946, b), the variances of the means of the groups of values for glucose utilisation in Table 28 were found to differ significantly ($0.05 > P > 0.02$). An approximately proportional relationship between the

Figure 40

The Relation Between the Standard Deviation and the Mean of Estimates of Glucose Utilisation.



The data are taken from Table 28 for hearts perfused without insulin. A similar relation is observed when the data of Table 29 are plotted.

TABLE 29

THE RATE OF GLUCOSE UTILISATION IN THE ABSENCE OF INSULIN BY HEARTS WHICH PRODUCED NO DETECTABLE LACTATE

The groups are those of Table 28 with the elimination of results from experiments in which lactate formation was detected. The data remaining from Groups 4 and 5 were pooled. Values are given for the means of the groups and the standard deviation from the mean.

Group	Concentration of perfusate glucose mg./100 ml.	Utilisation of glucose mg./g. true solid/hour	Number of experiments
1	93.1 ± 2.1	12.5 ±7.0	3
2	43.2 ± 3.0	15.8 ± 6.8	5
4, 5	21.0 ± 1.4	18.4 ± 9.6	3
6	16.9 ± 1.5	13.0 ± 3.8	5
7	12.6 ± 0.7	8.9 ± 6.0	5
8	9.6 ± 0.6	9.7 ± 3.3	4
9	7.3 ± 0.2	8.3 ±3.1	3
10	6.0 ± 0.5	8.1 ±2.0	4
11	4.3 ± 0.5	7.3 ± 0.6	4

magnitudes of the mean rates of utilisation and their standard deviations is apparent from Fig. 40 in which the intercept of a linear regression line does not differ significantly from zero.

The form of the relationship between the estimates of the rate of glucose utilisation and their error is of considerable importance in the determination of the parameters of utilisation. Different estimates of Km and Vmax can be calculated from the same set of experimental data because of changes in the effective statistical weight given to each point which occur when one of the first-order transformations of equation (7), (p.151), is fitted to the data, (Dowd and Riggs, 1965). In any analysis, the data should be ascribed significance or weight in inverse proportion to their relative errors. For this work there is therefore merit in the use of the method of Cohen (1968) whereby the hyperbolic relation (equation (7), (p.151)) is fitted directly and iteratively to the experimental data so as to minimise the sum of the squares of the relative errors of the dependent variable, rather than of the absolute errors.

Values for the parameters of utilisation were calculated from the individual data of Table 28 as well as from the means of the groups of data. In addition, the parameters were calculated after the elimination of those results associated with a detectable rate of lactate formation. Again, the data were treated both individually and in groups. In the latter case, the values used in the calculations were the means of the groups in Table 28 after the elimination of the appropriate data, except that the three results which remained from two of the groups were combined. The values are given in Table 29. Estimates of the parameters of utilisation were calculated by

TABLE 30

EFFECT OF THE METHOD OF CALCULATION ON THE ESTIMATES OF THE PARAMETERS OF GLUCOSE UTILISATION IN THE ABSENCE OF INSULIN

Relationship investigated:—			V vs S^x	1/V vs 1/S	V/S vs V
Unselected data of Table 28	Treated individually	K	10.6	4.6	-0.2
		V	21.9	12.9	13.1
	Treated in groups	K	10.8	8.0	7.6
		V	22.0	18.8	19.4
Data of Table 28 associated with low rate of lactate formation	Treated individually	K	6.5	2.9	0.3
		V	17.0	11.0	11.5
	Treated in groups	K	6.6	5.6	5.3
		V	17.1	15.8	16.0

x By the method of Cohen (1968).

K is expressed as mg./100 ml.

V is expressed as mg./g. true solid/hour.

TABLE 31

ESTIMATES OF THE PARAMETERS OF THE PERMEATION OF MONOSACCHARIDES IN RAT CARDIAC MUSCLE

Monosaccharide	Reference	Insulin mU./ml.	K_1 mM.	V_1 mMoles/g. true solid/ hour	K_2 mM.	V_2 mMoles/g. true solid/hour
D-glucose	This work	0	0.36	0.094	0.028	0.33
		100	1.11	0.440	0.028	0.33
D-glucose	Post, Morgan and Park (1961)	0	8.72	0.61	0.54	0.48
		100	27.78	3.00	0.54	0.48
D-Xylose	Gilbert (1963)	0	0.17	0.26	--	--
		0.2	5.49	0.19	--	--
		1.0	6.69	0.89	--	--
		4.0	7.08	5.41	--	--
		20.0	6.42	39.19	--	--
L-Arabinose	Gilbert (1963)	0	0.05	0.39	--	--
		0.2	1.41	0.20	--	--
		1.0	1.23	0.74	--	--
		4.0	2.23	5.56	--	--
		20.0	1.62	29.81	--	--

K_1, V_1 and K_2, V_2 designate respectively the parameters of permeation and the parameters of phosphorylation.

Values for K_1 and V_1 for the permeation of glucose in the absence of insulin, for this work, were taken from Table 30: K_1 = 6.5 mg./100 ml.; V_1 = 17.0 mg./g. true solid/hour.

In correcting values of other authors to the chosen units, there were taken to be 3.0 ml. of intracellular water per g. true solid and the ratio of wet to dry weight was taken to be 6.0.

Cohen's method and from the coefficients of unweighted linear regressions of $^1/v$ and $^1/x$ and of $^v/x$ on v. These estimates are presented in Table 30.

It is apparent from Table 30 that the greatest consistency between the estimates of the parameters of utilisation from the individual data and from the grouped data was given by Cohen's method. The different methods of estimation only gave comparable values when the data were grouped.

With the assumptions that in the absence of insulin the parameters of the utilisation of glucose approximate to those of the permeation of cardiac muscle by the sugar and that the best estimates of those parameters are derived from data obtained from hearts whose rate of lactate formation was low, K_1 for glucose permeation was of the order of 6.5 mg./100 ml. and V_1 approximated to 17 mg./g. true solid/hour. The line drawn in Fig. 39 passes through points calculated from equation (3) p.149 for the model system of a freely reversible carrier-mediated permeation of the cardiac cells in which glucose may be phosphorylated in an irreversible enzymic reaction. The parameters of phosphorylation were assumed to be unaffected by insulin; that is K_2 was taken to be 0.5 mg./100 ml., V_2 60 mg./g. true solid/hour, K_1 6.5 mg./100 ml. and V_1 17 mg./g. true solid/hour. The line is in reasonable accord with the experimental data.

The most probable values for the parameters of permeation and phosphorylation in the presence and absence of insulin are summarised in Table 31. Their significance is discussed in the following chapter.

CHAPTER FOUR

DISCUSSION

The estimates which are presented in Table 31 of the parameters of the process by which glucose permeates the cells of rat cardiac muscle conform with the observations that high concentrations of insulin raise both the K and the V for the permeation of D-glucose (Post et al., 1961) and of D-xylose and L-arabinose (Fisher and Zachariah, 1961; Gilbert, 1963). However, the primary objective of this work was not to confirm qualitatively an effect, but to make a quantitative study.

Normal variation among animals may be expected to be a preponderant source of error in the experiments reported here. Biological variation might have a greater effect on V, which might reflect the amount of the hypothetical carrier, than on K, which might reflect the character of the carrier. However, the K for glucose utilisation in the absence of insulin is apparently close to the lowest concentration of perfusate glucose which was convenient to use. Consequently, the experimental conditions were inappropriate for the precise estimation of the K of glucose utilisation, whatever may have been the impact of biological variation. On the other hand, an estimate was obtained by multiple regression (Table 27), for the K of glucose permeation, in the presence of insulin, with an error of approximately 15% but, in this case, the attribution of the estimate to the

process of permeation must be justified. A discussion of the errors associated with estimates of the parameters of glucose permeation is of limited relevance when the significance of the estimates depends on the validity of the assumptions which were the basis of their determination.

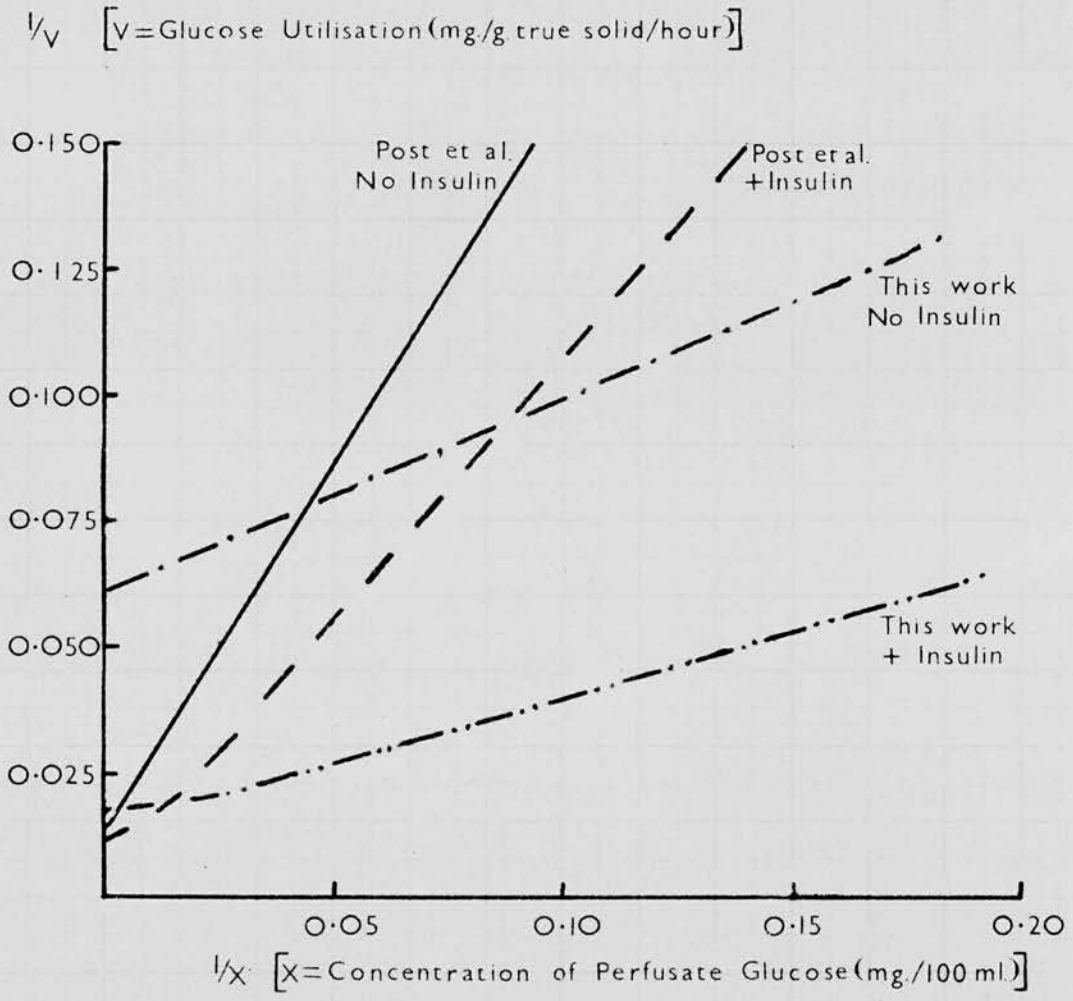
There are two crucial assumptions, other than those inherent in the hypothesis of carrier-mediated permeation. They are the assumption that there is no significant difference in glucose concentration between the perfusate and the interstitial water of the perfused heart, and the assumption that the kinetics of glucose utilisation in the absence of insulin approximate to those of permeation. The arguments upon which the first assumption was based were developed in the introductory chapter to Section III. They were sufficiently inconclusive to give pause to the ready acceptance of so fundamental an assumption. As was demonstrated on p.158 , Fig. 38, the form of the relationship between the reciprocals of glucose utilisation and of the concentration of perfusate glucose which is predicted by the model of reversible carrier-mediated permeation coupled to irreversible phosphorylation is similar to that predicted when diffusion of a substrate to an enzyme in part limits the rate of reaction. Both membrane limited permeation and an extracellular concentration gradient might combine to produce the experimental observations. As previously indicated, errors in the determination of the parameters of permeation which arise from neglecting the effect of an extracellular gradient in the concentration of glucose, lead to an overestimate of the half-saturation constant of permeation.

A comparison with estimates of the parameters of permeation which were obtained by methods other than that used in this work would be helpful in

Figure 41

PROPERTIES OF A MODEL OF GLUCOSE UTILISATION

A Comparison of the Conclusions of Post et al.(1961) and of This Investigation



The parameters of glucose permeation and phosphorylation in the presence and absence of insulin (given in Table 30) were substituted in the model for carrier-mediated permeation and irreversible phosphorylation.

assessing the impact of an extracellular gradient of glucose concentration on the kinetics of utilisation. Unfortunately, the only other investigation (Morgan, Henderson et al. 1961) of the parameters of glucose permeation in rat cardiac muscle is open to question on several counts, which were elaborated in the General Introduction. The criticism of that work, which has the most relevance to the investigation of the kinetics of glucose permeation in the presence of insulin, is that considerable error must have been associated with the estimates of the concentration of intracellular glucose.

Morgan, Henderson, et al., expressed their estimates of the rates of glucose utilisation and permeation in terms of the weight of the wet cardiac tissue. A relationship between the weight of cardiac muscle when wet and when dry is difficult to obtain with precision, because of variation in the amount of perfusate associated with the wet heart, whatever methods are employed for blotting (Gilbert, 1963). However, an approximate ratio of the weights of wet and dried hearts is 6:1. After adjustment by this factor, the values derived by Post et al. (1961), from the studies of Morgan, Henderson et al (1961), for the parameter of glucose permeation and phosphorylation in the presence of insulin were, respectively, K_1 = 500 mg./100 ml., V_1 = 540 mg./g. dry weight/hour, and K_2 = 9.7 mg./100 ml., V_2 = 87 mg./g. dry weight/hour. Apart, perhaps, in the case of the estimate of the maximum rate of phosphorylation, the agreement between these results and those obtained in this work is negligible. The validities of the two sets of parameters have been assessed (Fig. 41) by comparing the behaviour of the model system of reversible carrier-mediated permeation and irreversible phosphorylation, which each set of estimates predicts, with the experimental

observations. Clearly, if the model system, which was also used by Post et al. is meaningful, and the experimental observations of this work are not artefactual, the estimates of the parameters by Post et al. must be the more erroneous, because any overestimate of the effective extracellular concentration of glucose would increase the slope of a plot of $^{1}/v$ against $^{1}/x$. Although the previous estimate of the parameters of permeation appear to have been improved upon in this work, the possibility of error remains considerable.

In these circumstances, it is still possible to make comparisons with the results of studies of the permeation of the cardiac muscle of the rat by non-metabolised sugars. The inhibition of the permeation of these sugars and glucose by one another indicates that they share a common carrier. Two investigations (Fisher and Zachariah, 1961, and Gilbert, 1963) with D-xylose and L-arabinose as the permeants yielded results which were in fair accord. For comparison, the results of this work and that of Gilbert are given in the same units in Table 31. The use of molar quantities has obvious advantages when different sugars are to be compared. For the conversion, there were taken to be 3.0 ml. of intracellular water per g. true solid (Gilbert, 1963).

A comparison of the estimates of the parameters of the permeation of D-glucose, D-xylose and L-arabinose in the presence of insulin, is made less reliable by the very different concentrations of insulin which were used for glucose and for the other sugars. However, the half-saturation constants for D-xylose and L-arabinose are, within experimental error, independent of the concentration of insulin. If this independence extends to a concentration

of 100 mU of insulin per ml., the half-saturation constant which has been determined for glucose in this work is in no way incompatible with those of the non-metabolised sugars. Of course, similarity in the magnitude of the half-saturation constants for different sugars has no particular significance whereas the maximum rate of permeation for sugars which share a common carrier should be identical if the rate constants for the transfer of carrier and sugar-carrier complex are identical. No such identity can be detected between the maximum rates of permeation of glucose and of the non-metabolised sugars, if it is supposed that the action of insulin is the same at high concentrations as at low. It would seem possible that the V_{max} for glucose permeation has been underestimated.

However, it is probable that considerable errors are associated with the estimates which Gilbert made by following the time-course of permeation of the non-metabolised sugars and examining the linear relationship between f/t and $-\frac{\ln(1-f)}{t}$. From:-

$$\frac{f}{t} = -\frac{K+x}{x} \cdot \frac{\ln(1-f)}{t} - \frac{KV}{x(K+x)}$$

where f is the fractional penetration, x the extracellular sugar concentration, t the time of permeation, and K and V are the parameters of permeation: the coefficient, b, of $-\frac{\ln(1-f)}{t}$ is given by:

$$b = \frac{K+x}{x} \quad \text{whence } K = x\,(b-1)$$

Thus the accuracy of the estimate of K, and consequently of V, is determined partly by the magnitude of the difference of b from unity. In practice, the difference was small and of the same order as the standard

deviation of b. It can be shown that the apparent values of K and V are very sensitive to the alteration of one of the six estimates of f, which were made at each concentration of insulin, by the standard error of its mean. This sensitivity is particularly evident at high concentrations of insulin. Consequently, the accuracy of the individual estimates of the parameters of permeation of D-xylose and L-arabinose is sufficiently small for it to be only the consistency of the estimates as a set which gives them credence.

Further evidence on the reliability of the estimates of the K_m and V_{max} for the permeation of glucose in the presence of insulin can be obtained from a consideration of the comparability of the estimates which were obtained in the absence of the hormone, because different assumptions were involved in the two determinations. Although the possibility of the existence of an extracellular barrier to the diffusion of glucose is unlikely to be affected by the presence or absence of insulin, the lack of a significant departure from linearity in a plot of $^1/v$ against $^1/x$ (Fig. 39) suggests that the effect of any barrier can safely be neglected when hearts were perfused without insulin. The same linearity was taken to justify the assumption that the kinetics of glucose utilisation, in the absence of insulin, approximate to those of permeation. In Table 31, the estimates of the parameters of glucose permeation, which were made on this basis, are presented together with those obtained by Post et al. (1961). Substitution of the latter values in the model of reversible carrier-mediated permeation coupled to irreversible phosphorylation leads to the prediction of relationships which bear little resemblance to those observed experimentally in this work, (Fig. 41).

Probably a major cause of the discrepancies between the estimates in Table 31 is the likelihood that endogenous insulin influenced the rate of glucose utilisation during the period when Morgan, Henderson et al. made their measurements. Certainly, the rates of glucose utilisation, which were determined in the absence of exogenous insulin in both investigations, are only similar at low concentrations of perfusate glucose. The estimates, which were made by Fisher and Zachariah (1961) and Gilbert (1963), of the parameters of the permeation of non-metabolised sugars into rat cardiac muscle provide again the only alternative basis for comparison.

The relevant data for a comparison between this work and that of Gilbert are given in Table 31. A four-fold variation in the V of permeation in the absence of insulin is evident. Variation of this magnitude is perhaps admissible, but the assumption that the concentration of intracellular glucose is so low that the efflux of glucose from the cells can be discounted must lead to an underestimate of the true rate of permeation of glucose. It is most improbable that endogenous insulin influenced the permeation of the non-metabolised sugars, because Gilbert studied this process in hearts which were perfused for 40 minutes before being exposed to the sugar.

In the absence of insulin similar values have been obtained for the half-saturation constants and the maximum rates of permeation of D-glucose, D-xylose and L-arabinose into rat cardiac muscle. The method by which Gilbert obtained his results for the pentoses was the same whether permeation was studied in the presence of insulin or not, whereas, in this work, the data for glucose were analysed in two different ways. It might be supposed that the greater disparity between the estimates of the rate of

permeation of glucose and of the non-metabolised sugars, when these were studied in the presence of insulin, is a consequence of the analysis used in this work. A comparison with previous investigations on the permeation of non-metabolised sugars might therefore suggest that the assumption of a uniform concentration of extracellular glucose is invalid.

Lack of consideration of the effects of an extracellular gradient in glucose concentration must result in the overestimate of the half-saturation constant for permeation. Evidence of the likelihood of such an overestimation can be derived from the properties of the perfused heart during its approach to a stable metabolic state. As was discussed in Section II, Chapter Three, an initial decline in the rate of glucose utilisation was observed consistently only when the concentration of perfusate glucose exceeded some 20 mg./100 ml. The decrease in utilisation was attributed to the disappearance of endogenous insulin, which appears to be effective in promoting the utilisation of glucose only at the higher concentrations of the sugar. Such a phenomenon is explicable if it is assumed that endogenous insulin modifies only a fraction of the total carrier and that the properties of the modified and unmodified carriers are such that, at high concentrations of glucose, the modified carrier is predominant in effecting net permeation, whereas at low concentrations, the unmodified carrier predominates or both forms are equally effective.

The adequacy of this explanation can be examined with an elaboration of the model which was employed in the analysis of the kinetics of glucose utilisation in the presence of insulin. In this elaboration, the total amount of carrier is presumed to be divided, in any proportion, between

the form which has been modified by insulin and the unmodified form. There are therefore two carrier systems which work in parallel. Each form of the carrier has its characteristic half saturation constant but the maximum rate of permeation, which can be supported by each carrier, depends on the amount of the total carrier which is assumed to be modified by insulin. The kinetics of the metabolism of glucose were again taken to be those of an essentially irreversible enzyme-catalysed phosphorylation. This system can be described by a quadratic equation in x, the extracellular concentration of glucose, which is derived in Appendix 2. With this equation, the concentration of extracellular glucose, which is necessary to support a given rate of glucose utilisation or net permeation, can be calculated. A knowledge of the intracellular glucose concentration from the rate of utilisation and the parameters of phosphorylation is implicit. The contribution of each form of carrier to the net inward permeation can be readily calculated from the ascribed values for the parameters of permeation.

The proportion of the hypothetical carrier which is modified by endogenous insulin is a matter for speculation. It must be sufficient to account for the observed rate of glucose utilisation at the initiation of perfusion at a high concentration of perfusate glucose. In these circumstances, the rate of net inward permeation was as great as 50 mg./g. true solid/hour while the maximum rate which can be supported by the wholly unmodified carrier is about 15 mg./g. true solid/hour. The higher rate of net permeation and, therefore, of utilisation requires a higher concentration of intracellular glucose, which in turn must increase the degree of saturation of the unmodified carrier at the inner face of the membrane, thus reducing

TABLE 32

THE EFFECT OF INSULIN ON GLUCOSE UTILISATION IN A MODEL SYSTEM OF CARRIER-MEDIATED PERMEATION AND IRREVERSIBLE PHOSPHORYLATION WHEN AN INSIGNIFICANT FRACTION OF THE CARRIER IS ASSUMED TO BE MODIFIED

The parameters of the unmodified fraction are taken as: K_1' 5 mg./100 ml.; V_1' 15 mg./g. true solid/hour.

The parameters of phosphorylation are taken as: K_2 0.5 mg./100 ml.; V_2 60 mg./g. true solid/hour.

In the absence of insulin, the parameters of permeation are taken as insignificantly different from those of the unmodified fraction.

Parameters of the insulin-modified fraction	Concentration of extracellular glucose mg./100 ml.	Net permeation by modified fraction mg./g. true solid/hour	Net permeation by unmodified fraction mg./g. true solid/hour	Utilisation with insulin mg./g. true solid/hour	Utilisation without insulin mg./g. true solid/hour
V_1'' 50 mg./g. true solid/hour	0.44	0.83	1.17	2	1.20
K_1'' 25 mg./100 ml.	1.21	2.22	2.78	5	2.80
	2.83	4.88	5.12	10	5.30
	11.91	15.43	9.57	25	10.20
	219.14	40.34	9.66	50	14.20
V_1'' 50	0.56	0.54	1.46	2	1.45
K_1'' 50	1.60	1.50	3.50	5	3.55
	4.01	3.62	6.38	10	6.50
	20.11	13.99	11.01	25	11.70
	289.66	40.26	9.75	50	14.20
V_1'' 50	0.65	0.32	1.68	2	1.70
K_1'' 100	1.95	0.93	4.07	5	4.10
	5.39	2.51	7.49	10	7.55
	35.28	12.86	12.14	25	12.75
	479.66	40.16	9.85	50	14.40

TABLE 32 (cont.)

Parameters of the insulin-modified fraction	Concentration of extracellular glucose mg./100 ml.	Net permeation by modified fraction mg./g. true solid/hour	Net permeation by unmodified fraction mg./g. true solid/hour	Utilisation with insulin mg./g. true solid/hour	Utilisation without insulin mg./g. true solid/hour
V_1'' 100	0.31	1.17	0.83	2	0.90
K_1'' 25	0.82	3.01	1.99	5	2.05
	1.80	6.32	3.68	10	3.85
	5.96	17.84	7.16	25	7.95
	26.52	42.38	7.62	50	12.25
V_1'' 100	0.44	0.84	1.16	2	1.20
K_1'' 50	1.19	2.24	2.76	5	2.80
	2.74	4.99	5.01	10	5.20
	10.02	15.99	9.01	25	9.70
	43.14	41.56	8.44	50	13.00
V_1'' 100	0.56	0.54	1.46	2	1.45
K_1'' 100	1.59	1.52	3.48	5	3.50
	3.94	3.69	6.31	10	6.45
	17.27	14.37	10.63	25	11.25
	76.55	40.92	9.08	50	13.60

TABLE 33

THE EFFECT OF INSULIN ON GLUCOSE UTILISATION IN A MODEL SYSTEM OF CARRIER-MEDIATED PERMEATION AND IRREVERSIBLE PHOSPHORYLATION WHEN A SIGNIFICANT FRACTION OF THE CARRIER IS ASSUMED TO BE MODIFIED

The parameters of the unmodified fraction are taken as: K_1' 5 mg./100 ml.; V_1' 10 mg./g. true solid/hour.
In the absence of insulin, the parameters of permeation are: K_1 5 mg./100 ml.; V_1 15 mg./g. true solid/hour.
The parameters of phosphorylation are taken as: K_2 0.5 mg./100 ml.; V_2 60 mg./g. true solid/hour

Parameters of the insulin-modified fraction	Concentration of extracellular glucose mg./100 ml.	Net permeation by modified fraction mg./g. true solid/hour	Net permeation by unmodified fraction mg./g. true solid/hour	Utilisation with insulin mg./g. true solid/hour	Utilisation without insulin mg./g. true solid/hour
V_1'' 50 mg./g. true solid/hour	0.55	1.04	0.96	2	1.5
	1.51	2.77	2.23	5	3.4
K_1'' 25 mg./100 ml.	3.56	6.04	3.96	10	6.1
	15.13	18.15	6.85	25	11.0
	588.65	43.42	6.58	50	14.4
V_1'' 50	0.75	0.73	1.27	2	1.9
K_1'' 50	2.19	2.05	2.95	5	4.4
	5.58	4.92	5.08	10	7.7
	27.11	17.22	7.78	25	12.3
	545.95	43.42	6.58	50	14.4
V_1'' 50	0.94	0.46	1.54	2	2.3
K_1'' 100	2.93	1.40	3.60	5	5.4
	8.55	3.89	6.11	10	9.2
	50.37	16.57	8.43	25	13.2
	828.14	43.39	6.61	50	14.5

TABLE 33 (cont.)

Parameters of the insulin-modified fraction	Concentration of extracellular glucose mg./100 ml.	Net permeation by modified fraction mg./g. true solid/hour	Net permeation by unmodified fraction mg./g. true solid/hour	Utilisation with insulin mg./g. true solid/hour	Utilisation without insulin mg./g. true solid/hour
V_1'' 100	0.36	1.36	0.64	2	1.0
K_1'' 25	0.95	3.49	1.51	5	2.4
	2.07	7.26	2.74	10	4.3
	6.77	19.91	5.09	25	8.4
	29.21	44.80	5.20	50	12.4
V_1'' 100	0.55	1.05	0.95	2	1.4
K_1'' 50	1.49	2.80	2.20	5	3.4
	3.39	6.15	3.85	10	5.9
	11.97	18.61	6.39	25	10.3
	48.11	44.28	5.72	50	13.2
V_1'' 100	0.75	0.73	1.27	2	1.9
K_1'' 100	2.16	2.07	2.93	5	4.4
	5.39	5.01	4.99	10	7.6
	21.79	17.53	7.47	25	11.8
	86.29	43.88	6.12	50	13.7

the contribution of this fraction of the total carrier to the net rate of inward permeation. Consequently, 50 mg./g. true solid/hour can be taken to be about the minimum rate of permeation which should be possible for the fraction of modified carrier. Clearly, this implies that if only a small fraction of the carrier is modified by endogenous insulin the maximum rate of permeation after complete modification by insulin would be very large. If the apparent ineffectiveness of endogenous insulin at low concentrations of perfusate glucose is to be explained by the low degree of saturation of the modified carrier under these circumstances, it must also be supposed that the fraction of the total carrier which is modified is small or that both forms of the carrier are equally effective at these concentrations because an initial increase in the rate of glucose utilisation would be otherwise anticipated. No conclusive evidence of such an increase has been obtained.

Table 32 portrays the properties of the model system when the fraction of the carrier which was assumed to be modified by insulin is so small that the V_{max} of the unmodified carrier is not significantly reduced from its value in the absence of insulin. For comparison the rate of glucose utilisation at the same concentration of perfusate glucose has been calculated for the insulin-free system. It can be seen that the most important factor in mimicing the ineffectiveness of the modified carrier at low concentrations of perfusate glucose is the magnitude of the half-saturation constant of that fraction. Substantially the same result emerges when a large fraction of the total carrier is assumed to be modified by insulin (Table 33). However, whereas in the former case the contribution of the

modified carrier to net permeation at low concentrations of perfusate glucose decreases with increasing K, in the latter, the equal effectiveness of the modified and unmodified fraction in facilitating the permeation of glucose makes the influence of endogenous insulin even less marked. Both approaches indicate that the effect of perfusate glucose concentration on the time-course of glucose utilisation by hearts perfused without exogenous insulin is explicable if the half-saturation constant for the permeation of glucose in the presence of insulin is greater than 25 mg./100ml.

These arguments are based on assumptions which are independent of the problem of the magnitude of the gradient in the glucose concentration in the extracellular water. It is concluded that there is no significant difference between the concentrations of perfusate and interstitial glucose and that K for the permeation of glucose has not therefore been overestimated.

The problem of the reliability of the estimates of the parameters of glucose permeation in the presence of insulin therefore reduces to the lack of accord with the work of Gilbert on non-metabolised sugars. It may be noted that a value of 100 mg./100 ml. for the half-saturation constant for the permeation of glucose must be associated with a maximum rate of permeation of approximately 3500 mg or 20 mMoles/g. true solid/hour if the slope of the plot of $^{1}/v$ against $^{1}/x$ in Fig. 34 is to be in agreement with the experimental relationship at low concentrations. These values would concur not only with those of Gilbert (Table 31) but also with the characteristic time course of glucose utilisation. However, it will be recalled that the half-saturation constant for permeation was determined independently of the maximum rate of permeation as the reciprocal of one of the coefficients of a multiple

regression (p.156), and found to be 23 mg./100 ml. On the other hand, inspection of Tables 32 and 33 will suggest that a K for glucose permeation in the presence of insulin of 23 mg./100 ml. does not account adequately for the form of the time-course of glucose utilisation in the absence of insulin at different concentrations of perfusate glucose.

The conflicts of these conclusions might be mitigated if there were no basis for assuming that glucose and other sugars have the same maximum rate of permeation in muscle. If the rate constants for the transfer across the cell membrane of carrier and the various sugar-carrier complexes were not identical disparities in the maximum rate of permeation by the sugars would be expected. The possibility exists that unreal comparisons have been made. Despite their undoubted inaccuracy, the values which are given from this work in Table 31 for the parameters of glucose permeation in rat cardiac muscle in the presence and absence of insulin are claimed to represent an improvement over previous estimates.

CHAPTER FIVE

SUMMARY

1. Estimates are presented of the parameters for the permeation of glucose into rat cardiac muscle in the presence and absence of insulin.
2. The utilisation of glucose in the presence of insulin is analysed in terms of a model system of reversible carrier-mediated permeation and irreversible phosphorylation. The properties of the model are in accord with experimental observations. From graphical exploration and the regression coefficients of a multivariate relationship predicted by the model, estimates of the parameters of permeation are derived. However, the observed relationship between utilisation and the concentration of perfusate glucose is also explicable by a concentration difference between perfusate and interstitial glucose.
3. The effect of perfusate glucose concentration on the time-course of glucose utilisation in the absence of insulin was analysed in terms of partial modification of the carrier by endogenous insulin. The analysis suggests that the estimates of the parameters of permeation are of the correct order and that a gradient in the concentration of extracellular glucose cannot have significantly affected the observations.
4. The utilisation of glucose in the absence of insulin was assumed to be limited by permeation at all concentrations of perfusate glucose and the kinetics of the process taken to approximate to those of permeation. Estimates of the parameters of permeation were obtained from the hyper-

bolic relationship between utilisation and concentration of perfusate glucose.

5. Previous estimates of the parameters of glucose permeation differ irreconcilably from those found in this work.
6. Estimates of the parameters of permeation of non-metabolised sugars are in partial, though not complete, accord with those attributed here to glucose.

GENERAL DISCUSSION

The object of this work - the investigation of the kinetics of the permeation of glucose into the cells of rat cardiac muscle and of the effect of insulin thereon - has been less than perfectly achieved. Although the original approach to the objective through the determination of the concentrations of both intracellular and extracellular glucose, when in a steady-state relationship, proved impracticable, the alternative of the analysis of the kinetics of glucose utilisation was comparatively rewarding. In this last respect, considerable success has met the application of the hypothesis of carrier-mediated permeation.

The hypothesis met two tests when it was used to interpret the kinetics of glucose utilisation in a steady state and the time-courses of utilisation by hearts perfused at various concentrations of glucose but without exogenous insulin. Subtleties may be concealed by the variations inherent in the experimental material, but the carrier-hypothesis in its simplest form is sufficient to account for the phenomena which were observed in this work.

Both tests of the carrier hypothesis were possible largely because of the characteristics of the system for cardiac perfusion in a steady state, which has been developed. In this area of technique, the investigation has been most fruitful. The ability to study cardiac metabolism under constant and well-defined conditions has potential applications other than that which has been described here. Departure from a steady state, after a modification

in the conditions of an experiment, should be more readily detected than variations in the rate of change of an inconstant environment. Modifications of the experimental conditions might involve the introduction of an additional metabolite, hormone or pharmacological agent. Indeed, the effect of any change in the composition of the perfusate could be investigated without interference by simply changing the infusate. The impact on a steady state of cardiac metabolism might also be a more sensitive indicant of the effect of changes in the physical conditions of perfusion pressure, temperature and oxygenation. For such investigations, a particularly useful facility might be the determination of the time-course of utilisation of a metabolite, which would clarify any changes in the properties of a heart during the approach to and departure from a steady state. The system of cardiac perfusion which was developed for this work therefore appears to be superior in some respects to a system of fully closed-circuit perfusion. A greater quantity of information is provided by the determination of a time-course of utilisation and it might reasonably be claimed that the quality of the information is also superior because of the better definition of the conditions of the experiment.

Although possessing some notable advantages, the technique for cardiac perfusion in the steady state is not without limitations. Restrictions on the use of the technique all result from the accuracy of an estimate of the rate of utilisation of a metabolite being dependent on the errors associated with the measurement of the difference between the concentration of the substance in the perfusate and in the infusate. Despite the use of a precise method for the estimation of glucose, it was nevertheless impossible to

perfuse hearts, without insulin, at a concentration of perfusate glucose greater than 100 mg./100 ml. without incurring an error in the estimate of utilisation of about 10%. The upper limit to the practicable concentration of perfusate glucose led to difficulties in the determination of the concentration of intracellular glucose in hearts perfused without insulin. Exploratory efforts towards the estimation of the total cardiac glucose met with little success, but the amount of glucose, whose distribution was to be studied, was very small under the conditions when glucose utilisation could be measured most accurately. As was discussed in Section III Chapter Four, even with an accurate estimate of the total cardiac glucose, the possible sources of error in determining the concentration of intracellular glucose at low concentrations of perfusate glucose were considerable in hearts perfused without insulin. In mitigation of the upper limit imposed on the practicable concentration of perfusate glucose and the difficulties which ensue therefrom is the capacity for making accurate estimates of utilisation at a constant low concentration of perfusate glucose. In a closed perfusion system, the fractional fall in concentration of perfusate glucose is more rapid at low than at high concentrations of perfusate glucose for the same volume of perfusate. In these circumstances, the effective concentration of perfusate glucose is particularly difficult to define.

The estimation of utilisation in the steady state is impracticable when the difference in the concentration of the metabolite in the infusate and perfusate is small and the absolute magnitudes of the concentrations are large. Provided that the control of the perfusion pressure and temperature, oxygenation, and filtration of the perfusate remain satisfactory, reduction

of the volume of recirculated perfusate is the more desirable way of raising the upper limit to the concentrations, because reduction of the rate of infusion carries the penalty of prolonging the approach to a steady state. Because significant reductions in the volume of recirculated perfusate from the 6 ml. of this work will be made with difficulty, the technique for the estimation of utilisation in a steady state over a wide range of concentrations of the metabolite is most suited to conditions of rapid uptake of exogenous nutrient. The principle might therefore be applied usefully when the perfused heart performs external work.

The working heart presents metabolic problems and concepts of permeation which might profitably be examined by the perfusion technique described here. In some circumstances, glucose, whether accompanied by insulin or not, might be an inadequate source of nutrient for the working heart. The maximum rate of glucose utilisation by the basal preparation in the presence of insulin accounts well for the oxygen consumption, 39 µl/mg. dry weight/hour (Fisher and Williamson, 1961). The maximum rate of utilisation may therefore reflect the energy requirement. If the energy requirement is increased sufficiently in the working heart, glucose utilisation might be limited by the maximum rate of permeation, which, in this work, was found to be not much more than the maximum rate of utilisation. However, an effect of muscular work in increasing permeability to sugars in the perfused rat heart has been reported (Morgan, Neely, Wood, Liebecq, Liebermeister, and Park, 1965; Neely, Liebermeister and Morgan, 1967$_b$). The attribution of an increase in permeability of cardiac muscle to the effect of muscular work is open to doubt. Oxygen in solution in the perfusate may be in inadequate supply

for the working heart. Certainly the possibility of hypoxia is increased and it is significant that anoxia has been found to stimulate the permeation of sugars into the rat diaphragm (Randle and Smith, 1958), the perfused rat heart (Morgan, Randle, and Regen, 1959; Morgan, Henderson, et al., 1961) and the frog sartorius muscle (Ozand, Narahara and Cori, 1962). Randle and Smith (1960) suggested that a glucose carrier might exist in equilibrium with a phosphorylated form incapable of combining with glucose. The position of this equilibrium would be affected by the levels of adenosine triphosphate (ATP) in the cell and therefore by the energy requirement and aerobic state of the cell. Insulin might inhibit the formation or stimulate the breakdown of the phosphorylated carrier. However, in order to explain the effect of insulin on the parameters of permeation, this hypothesis is inadequate since it would predict only a change in the capacity to transport a sugar and not in the half-saturation constant. Only if it were supposed that the postulated phosphorylated form is capable of complexing with a sugar but is characterized by a lower half-saturation constant would this hypothesis fit the facts.

It would be tempting to extend the concept of a carrier system which is sensitive to the concentration of ATP to include a sensitivity to the concentration of adenosine 3',5'-phosphate (cyclic AMP). However, the level of this well documented mediator of hormone action (Sutherland, Øye, and Butcher, 1965) has not been found to change in cardiac muscle in response to insulin (unpublished observations cited by Robison, Butcher & Sutherland, 1968). Such negative evidence need not exclude a possible involvement of cyclic AMP when the basis of the action of insulin, anoxia and work remains

largely a matter for speculation. When it is recalled that β-hydroxybutyrate and octanoate have been reported to inhibit the efflux of L-arabinose from rat cardiac muscle, (Randle, Newsholme, and Garland, 1964), the permeability of the cells to sugars might be supposed to be regulated by some factor which reflects the metabolic state of the cells.

Hopefully, investigation of the kinetics of glucose utilisation in the basal and working heart which is perfused with glucose alone and with competing metabolites would add to an understanding of the permeability of cardiac muscle to glucose. Such an investigation would be the more fruitful if the significance of the extracellular gradient in glucose concentration were established.

Knowledge of the rate of diffusion of glucose across the capillary walls remains desirable for the full exploitation of a technique which offers the highest precision in estimates of rates of utilisation which are relatively high at low substrate concentrations in the perfusate - the conditions which favour a large extracellular gradient.

Appendix 1

The Kinetics of Permeation According to the Carrier Hypothesis

Let x be the concentration of extracellular permeant,

y " " " " intracellular permeant,

C' " " " " free carrier at the outer face of the membrane,

C'' " " " " " " " " inner face of the membrane,

PC' " " " " complexed carrier at the outer face of the membrane,

PC'' " " " " complexed carrier at the inner face of the membrane,

C " " " " carrier-free or complexed.

k_1 " " rate constant for complex formation

k_2 " " " " " " dissociation

k_3 " " " " " the transfer of free and complexed carrier across the membrane

In a steady state, when x, y, C', C'', PC' and PC'' are constant, the following relations hold:-

$$C' + C'' + PC' + PC'' = C \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

$$dPC'/dt = k_1 x C' + k_3 PC'' - (k_3 + k_2) PC' = 0 \quad .. \quad .. \quad .. \quad (2)$$

$$dPC''/dt = k_1 y C'' + k_3 PC' - (k_3 + k_2) PC'' = 0 \quad .. \quad .. \quad .. \quad (3)$$

$$dC'/dt = k_2 PC' + k_3 C'' - (k_3 + k_1 x) C' = 0 \quad .. \quad .. \quad .. \quad (4)$$

$$dC''/dt = k_2 PC'' + k_3 C' - (k_3 + k_1 y) C'' = 0 \quad .. \quad .. \quad .. \quad (5)$$

Summing (2) and (4) gives:-

$$- C' + C'' - PC' + PC'' = 0 \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Then, from (1) and (6):-

$$C' = C/2 - PC' \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (7)$$

and $C'' = C/2 - PC''$ .. .. (8)

Substituting for C' and C" in (2) and (3) gives:-

$$(k_3 + k_2 + k_1x)\, PC' - k_3\, PC'' = k_1\, C/2.\, x \quad .. \quad .. \quad .. \quad .. \quad (9)$$

and $-k_3\, PC' + (k_3 + k_2 + k_1y)\, PC'' = k_1\, C/2.\, y$ .. .. (10)

Eliminating PC', gives:-

$$PC'' = \frac{k_1 C}{2} \frac{k_3x + k_3y + k_1xy + k_2y}{(k_3 + k_2 + k_1x)(k_3 + k_2 + k_1y) - k_3^2} \quad .. \quad .. \quad .. \quad (11)$$

Now, the net influx of permeant, v, is given by:-

$$v = k_2PC'' - k_1yC''$$

and from (8):-

$$v = (k_2 + k_1y)\, PC'' - k_1\, C/2.y \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (12)$$

Substituting for PC":-

$$v = \frac{k_1 C}{2} \frac{k_3\, k_2\, (x - y)}{(k_3 + k_2 + k_1\, x)(k_3 + k_2 + k_1y) - k_3^2}$$

$$\therefore \quad v = \frac{k_2k_3}{k_1} \cdot \frac{C}{2} \cdot \frac{(x - y)}{\left(\frac{k_2 + k_3}{k_1} + x\right)\left(\frac{k_2 + k_3}{k_1} + y\right) - \frac{k_3^2}{k_1^2}} \quad .. \quad .. \quad .. \quad (13)$$

When $k_3 << k_2$, (13) approximates to:-

$$v \simeq \frac{VK\,(x - y)}{(K + x)(K + y)} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (14)$$

where $VK = \frac{k_2k_3}{k_1} \cdot \frac{C}{2}$

and $K = \frac{k_2 + k_3}{k_1}$

so that $V = \frac{k_2\, k_3}{k_2 + k_3}\, \frac{C}{2}$

Appendix 2

The Kinetics of Glucose Permeation and Phosphorylation in the Steady State when the Carrier is Partly Modified by Insulin

Let x be the concentration of extracellular glucose

y " " " " intracellular glucose

v " " rate of net influx and of phosphorylation

K_2 and V_2 be the Km and Vmax of enzyme-catalysed phosphorylation

K' and V' be the parameters of permeation for the unmodified carrier.

K'' and V'' " " " " " " " " modified carrier.

For glucose phosphorylation:

$$v = \frac{V_2 y}{K_2 + y} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

and

$$y = \frac{K_2 v}{V_2 - v} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

For glucose permeation:

$$v = \frac{K' V' (x-y)}{(K'+x)(K'+y)} + \frac{K'' V'' (x-y)}{(K''+x)(K''+y)} \qquad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

Rearranging (3):-

$$\left[\frac{K' V'}{K'+y} + \frac{K'' V''}{K''+y} - v\right] x^2 + \left[\frac{K' V' (K''-y)}{K' + y} + \frac{K'' V'' (K'-y)}{K''+ y} - v \ (K' + K'')\right] x$$

$$- K' K''\left(\frac{V' y}{K'+y} + \frac{V''y}{K''+y} + v\right) = 0 \qquad .. \quad .. \quad .. \quad (4)$$

Substituting for y from (2) and assuming values for the parameters of permeation and phosphorylation, and for the rate of glucose utilisation, the coefficients of equation (4) can be calculated. Thus the steady state

concentrations of extracellular and intracellular glucose, which support an assumed rate of glucose utilisation are given.

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